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14. ABSTRACT To discover the mechanisms of I3C/DIM action on prostate cancer cells, we have investigated the molecular effects of I3C/DIM on Akt and NF-κB pathways and their downstream genes which play critical roles in the control of cell survival. We have found that I3C/DIM significantly inhibited the activation of Akt in PC-3 and LNCaP prostate cancer cells. Moreover, we found that I3C/DIM also significantly inhibited the activity of NF-κB. However, we did not observe such inhibitory effects of I3C/DIM on non-tumorigenic CRL-2221 prostate epithelial cells. Most importantly, we found that I3C/DIM could inhibit NF-κB activation and AR transactivation in prostate cancer cells transfected with activated Akt. These results demonstrate that there is a crosstalk between Akt, NF-κB, and AR signaling pathways and I3C/DIM could interrupt the crosstalk, leading to the induction of apoptosis. From transfection experiments, we found that I3C/DIM inhibited the expression of IKKβ, the subsequent phosphorylation of IκBα, and the nuclear localization and activation of NF-κB. By microarray analysis, we found that I3C/DIM could regulate the expression of genes which control cell cycle, apoptosis, and signal transduction. I3C/DIM also inhibited angiogenesis and invasion. These results suggest that I3C and DIM may be potent agents for the prevention and/or treatment of prostate cancers.

15. SUBJECT TERMS
None provided.

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Introduction

This is the final progress report but our previous progress report was very comprehensive documenting progress in all tasks except the results of few minor experiments. In this report we are clearly showing the data in support of all tasks proposed under this grant proposal. Previous epidemiological and dietary studies have shown an association between high dietary intakes of vegetables and decreased prostate cancer risk (1). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family appears to be most effective at reducing the risk of cancers (2). Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family, and it is readily converted to its dimeric product, 3,3'-diinolylmethane (DIM). It has been found that I3C/DIM inhibit the carcinogens in animal experiments and the growth of various cancer cells in culture (3-5). The data from our laboratory and others have shown that I3C/DIM inhibit cell growth and induce apoptotic cell death in both androgen-sensitive as well as androgen independent prostate cancer cells (6-10), suggesting that I3C/DIM may be useful agents for prevention and/or treatment of hormone-refractory prostate cancer for which there is no curative therapy. However, the molecular mechanisms by which I3C/DIM exert their inhibitory effects on prostate cancer cells have not been fully elucidated. It has been reported that Akt, NF-κB, and AR pathways play critical roles in the control of cell survival and cellular response to cancer therapy. To discover the mechanisms of I3C/DIM action on prostate cancer cells, we have investigated the molecular effects of I3C/DIM on Akt, NF-κB, and AR signaling pathways. We have found that I3C/DIM significantly inhibited the activation of Akt under un-stimulated and stimulated conditions in PC-3, LNCaP, and C4-2B prostate cancer cells. Moreover, we found that I3C/DIM also significantly inhibited the DNA-binding activity of NF-κB in prostate cancer cells. However, we did not observe such inhibitory effects of I3C/DIM on non-tumorigenic CRL-2221 prostate epithelial cells, suggesting the selective inhibitory effects of I3C/DIM on prostate cancer cells. Most importantly, we found, for the first time, that I3C/DIM could inhibit NF-κB activation and AR transactivation in prostate cancer cells transfected with activated Akt. These results demonstrate that there is a crosstalk between Akt, NF-κB, and AR signaling pathways. I3C/DIM could inactivate Akt-related signaling molecules that are important regulators of NF-κB DNA-binding activity and AR transactivation, interrupting the crosstalk and leading to the induction of apoptotic process. To further investigate the molecular mechanism by which I3C/DIM inhibit NF-κB signaling, we conducted transfection experiments with expression vectors of MEKK, MEK, NIK, and IKK, which are known to play important roles in the activation of NF-κB, and tested the alteration in the expression of IKKβ, IκBα, p-IκBα, NF-κB, IKKα, and MEKK in parental and transfected PC-3 cells treated with I3C/DIM. We found that I3C/DIM inhibited the expression of IKKβ, the subsequent phosphorylation of IκBα, and the nuclear localization and activation of NF-κB, resulting in inhibition of cell growth and induction of apoptosis in prostate cancer cells. By microarray analysis, we also found that I3C/DIM could regulate the expression of genes, which are critically involved in the control of cell proliferation, cell cycle, apoptosis, Akt and MAPK signal transduction. These results suggest that I3C and DIM could be potent agents for the prevention and/or treatment of androgen sensitive and androgen-refractory prostate cancers. Because Akt, NF-κB, and AR pathways also control cellular response to cancer therapy, these observations provide important molecular evidence, which could be further exploited for sensitization of prostate cancer cells to commonly available therapeutics.

Body of Report

The original statement of work in the proposal is listed below:

Task-1: We will determine whether treatment of prostate epithelial cells with I3C/DIM elicit responses leading to modulation of NF-κB and investigate the molecular mechanism of NF-κB inactivation as proposed under specific aim-1. This investigation will be conducted using both I3C as well as DIM in androgen sensitive (LNCaP) and androgen independent (PC-3) prostate cancer cells and the data will be compared to those obtained from non-tumorigenic prostate epithelial cells (CRL2221). Task 1 will take 8 months to complete. Time Period 0-8 months.

Task-2: We will determine whether constitutive activation of Akt (by gene transfection studies) increases NF-κB activation in prostate epithelial cells as indicated under task-1, and thereby inhibits apoptotic processes induced by I3C/DIM. We will conduct transient transfection experiments followed by establishing stably transfected cells in the future in order to determine the inhibition of I3C/DIM induced cell death in those cells that over-express Akt and NF-κB. Task 2 will take 6-12 months to complete. Time Period 8-20 months.

Task-3: Once we complete task-1 and 2, we will start working on task 3, which will have significant number of transfection experiments. Task 3 will determine whether treatment of prostate epithelial cells with I3C/DIM will elicit responses that may determine causal association between the inactivation of Akt and NF-κB with the induction of apoptosis. Furthermore, we will investigate what signaling pathways are modified by I3C/DIM that leads to NF-κB inactivation. We will investigate different kinases that are involved in the NF-κB pathway, particularly the status of IKK, MAP kinase-MEK, MEKK1 and NIK. In order to establish cause and effect relationships of these genes with the ultimate effect of I3C/DIM, several transfection experiments are planned which will be very time consuming and labor intensive. Hence this task will take considerably more time. We expect to complete this task within 12 months. Time Period 20-32 months.

Task-4: Task-4 will be focused on to complete all data analysis, manuscript writing and for the development of novel ideas that may be submitted to Federal Agencies for further continued funding in a larger scale encompassing animal and human investigations to test (a) whether I3C/DIM could be an effective agent for the prevention and/or for the treatment of tumors in animal models, (b) whether I3C/DIM pre-exposure of prostate cancer cells will sensitize these cells to commonly available chemotherapeutic agents, and finally (c) whether I3C/DIM could be an effective agent for the prevention and/or treatment of human prostate cancer. Task 4 will be completed within the time period of 32-36 months.

We are now reporting the research accomplishments associated with task-1 to task-4 as outlined in the Statement of Work.

Task-1. Our task-1 was focused on investigating the effects of I3C and DIM on modulation of NF-κB and the molecular mechanism(s) responsible for NF-κB inactivation, anti-proliferation, and apoptosis caused by I3C and DIM in human prostate cancer cells. We investigated the effects of I3C and DIM on the regulation of cell growth, apoptosis induction, and NF-κB activity. We found that I3C and DIM inhibited cell growth, induced apoptosis, and inactivated NF-κB activity in PC-3 and LNCaP cells. However, such effect was not observed in non-tumorigenic CRL-2221 prostate epithelial cells.

1. I3C and DIM inhibited the growth of prostate cancer cells.

We treated PC-3 and LNCaP cells with 0-100 μM I3C or 0-60 μM DIM for 24, 48, and 72 hours. By MTT assay, we found that I3C and DIM significantly inhibited the growth of PC-3 and LNCaP prostate cancer cells. However, such effect was not observed in non-tumorigenic CRL-2221 prostate epithelial cells (Figure 1, 2, 3, and 4).

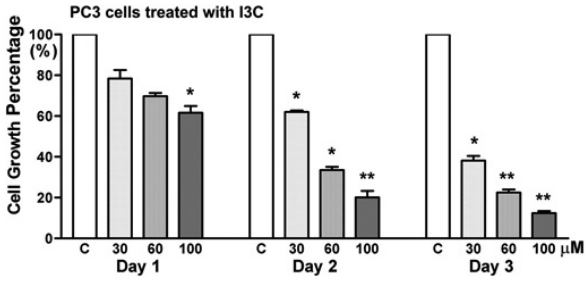


Figure 1: *MTT assay showing the significant inhibition of cell growth by I3C in PC-3 cells (*: p<0.05).*

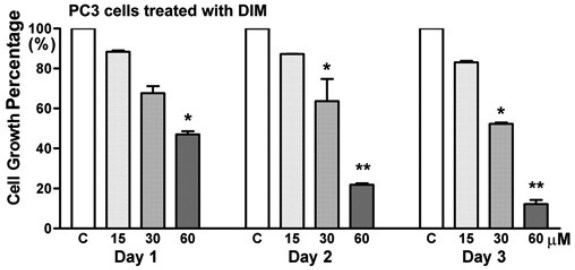


Figure 2: *MTT assay showing the significant inhibition of cell growth by DIM in PC-3 cells (*: p<0.05).*

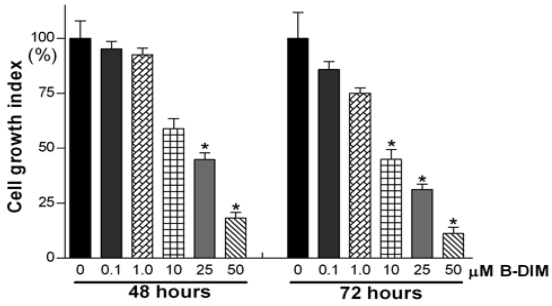


Figure 3: *MTT assay showing the significant inhibition of cell growth by DIM in LNCaP cells (*: p<0.05).*

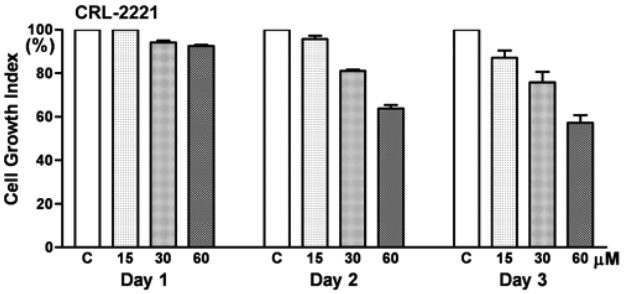


Figure 4: *MTT assay showing that there is no significant growth inhibition in CRL-2221 cells by DIM.*

2. I3C and DIM significantly induced apoptosis in prostate cancer cells.

We treated PC-3 and LNCaP cells with 0-100 μM I3C or 0-60 μM DIM for 24, 48, and 72 hours. By apoptosis assays including DNA ladder assay and ELISA, we found that I3C and DIM significantly induced cell apoptotic death in PC-3 and LNCaP prostate cancer cells. However, such effect was not observed in non-tumorigenic CRL-2221 prostate epithelial cells (Figure 5, 6, 7, and 8).

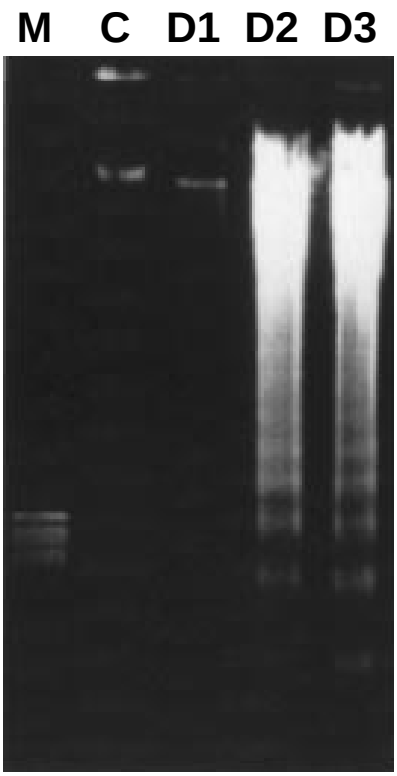


Figure 5: *DNA ladder analysis showing I3C induced apoptosis in PC-3 cells. M: DNA marker; C: control; D1 to D3: 60 μM I3C treatment for 1 to 3 days.*

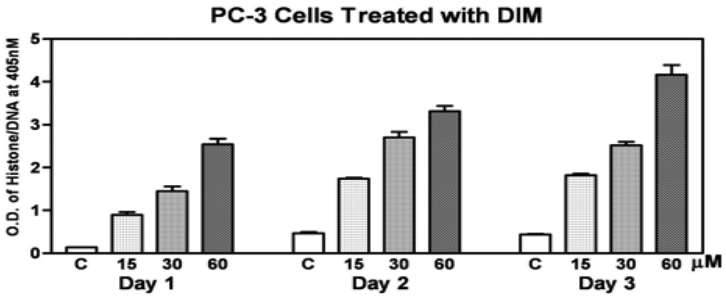


Figure 6: *ELISA showing the significant induction of apoptosis by DIM in PC-3 cells.*

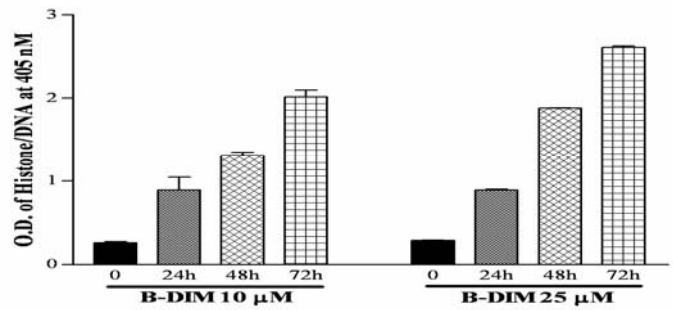


Figure 7: *ELISA showing the significant induction of apoptosis by DIM in LNCaP cells.*

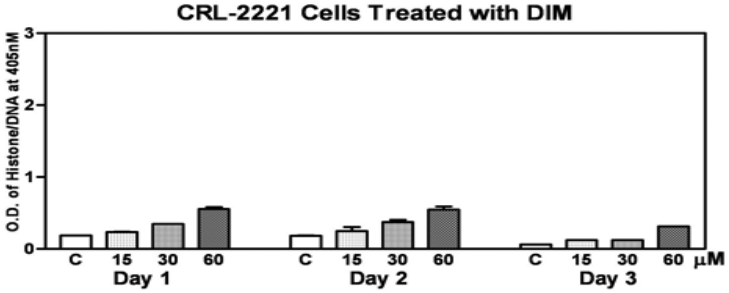


Figure 8: *ELISA showing no significant induction of apoptosis by DIM in CRL-2221 cells.*

3. I3C and DIM significantly inhibited the activity of NF-κB.

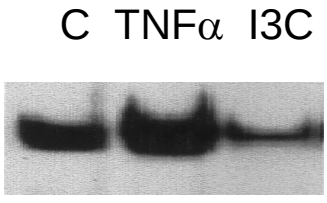


Figure 9. *EMSA showing that NF-κB was activated by TNFα and inactivated by I3C. C: control.*

We treated PC-3, LNCaP, and C4-2B cells with 60 μM I3C or 25 μM DIM for 24, 48, and 72 hours. The nuclear proteins from each sample were extracted and subjected to EMSA. We observed that both TNF-α and DHT induced activity of NF-κB as expected. More importantly, we found that I3C and DIM significantly inhibited the activity of NF-κB and abrogated the activation of NF-κB by DHT in PC-3 and LNCaP prostate cancer cells. However, such effect was not observed in non-tumorigenic CRL-2221 prostate epithelial cells (Figure 9 and 10).

Some of above data has been published in Oncogene (10).

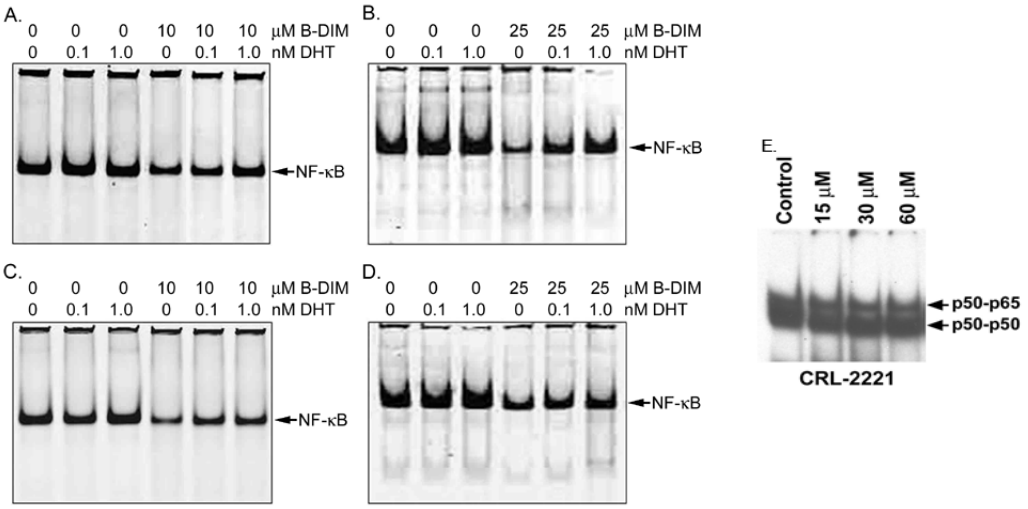


Figure 10. *EMSA showed that B-DIM inhibited basic and stimulated activity of NF-kB DNA binding activity in LNCaP (A and B) and C4-2B (C and D) cells. No such effect was observed in CRL-2221 cells (E)*

4. I3C and DIM regulated the genes related to NF-κB pathway and cell survival.

To investigate the molecular mechanisms of I3C and DIM effects on NF-κB inactivation, anti-proliferation, and apoptosis, we utilized the high-throughput microarray, which can monitor the expression of twenty two thousands of genes simultaneously and rapidly, to determine the alternation of gene expression profiles of PC-3 prostate cancer cells exposed to I3C and DIM. We have identified the genes that are involved in cell cycle arrest, apoptosis, and regulation of transcription factors including NF-κB affected by I3C and DIM treatment (Table 1). The results from microarray analysis have been presented in AACR Annual Meeting and published in The Journal of Nutrition [(11, 12); please see Appendix 1 and 3]. The summary of our results in the form of an abstract of our paper is presented below.

Abstract: Studies from our laboratory and others have shown that indole-3-carbinol (I3C) and its *in vivo* dimeric product 3,3'-diindolylmethane (DIM) inhibit the growth of PC-3 prostate cancer cells and induce apoptosis by inhibiting NF-κB and Akt pathways. In order to obtain a comprehensive gene expression profiles altered by I3C and DIM treated PC-3 cells, we have utilized cDNA microarray to interrogate 22,215 known genes by using Affymetrix Human Genome U133A Array. We found a total of 738 genes which showed >2 fold change after 24 hours of DIM treatment. Among these genes, 677 genes were down-regulated and 61 genes were up-regulated. Similarly, 727 genes showed >2 fold changes in the expression with down-regulation of 685 genes and up-regulation of 42 genes in I3C treated cells. The altered expressions of genes were observed as early as 6 hours and were more evident with longer treatment. Upon cluster analysis, we found that both I3C and DIM up-regulated expression of genes that are related to the Phase I and Phase II enzymes, suggesting their increased capacity for detoxification of carcinogens or chemicals. We also found that I3C and DIM down regulated the expression of genes, which are critically involved in the regulation of cell growth, cell cycle, apoptosis, signal transduction, Pol II transcription factor, and oncogenesis. Real-time RT-PCR analysis was conducted to confirm the data of cDNA microarray, and the results were consistent with the microarray data. From these results, we conclude that I3C and DIM caused changes in the expression of a large number of genes that are related to the control of carcinogenesis, cell

survival, and physiological behaviors. This may provide molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC-3 prostate cancer cells and these information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer.

Table 1. Fold changes of specific genes in PC-3 cells treated with 60 μmol/L I3C or 40 μmol/L DIM

Gene Name	I3C			DIM		
	6h	24h	48h	6h	24h	48h
Phase I & II enzyme						
NM_000499.2 cytochrome P450 (CYP1A1)	1.4*	3.7	5.2	1.8	2.2	5.4
M25813.1 Human P450c21 gene	NC**	NC	NC	NC	NC	3.5
N21019 cytochrome P450	NC	NC	NC	NC	5.2	3.5
NM_000104.2 cytochrome P450 (CYP1B1)	NC	NC	NC	2	1.9	2.2
NM_000853.1 glutathione S-transferase theta 1	NC	NC	2.1	NC	NC	2.1
NM_019886.1 carbohydrate sulfotransferase 7	NC	NC	NC	1.7	1.4	2
NM_012200.2 glucuronosyltransferase I	NC	NC	NC	12.6	NC	NC
AB009598 glucuronyltransferase I	NC	NC	NC	2.3	NC	NC
Cell growth, Cell Cycle, Apoptosis						
K03193.1 Human epidermal growth factor receptor	-1.4***	-2.6	-4.3	1	-2.7	-4.1
BF061658 transforming growth factor	NC	-1.8	-4	-1.2	-5.1	-6.4
NM_003238.1 transforming growth factor	NC	-2	-6.9	-1.2	-3.5	-4.9
NM_000358.1 transforming growth factor	-1	-1.1	-2.1	-1	-3.3	-3.7
M19154.1 Human transforming growth factor-beta-2	NC	-2	-5.4	-1	-3	-4.7
NM_002006.1 fibroblast growth factor 2	NC	-1.9	-4.9	NC	-1.8	-1.3
AI434345 activating transcription factor 1	-1.2	-1.4	-3.8	NC	-1.8	-2.2
Z24725.1 mitogen inducible gene mig-2	NC	-1.4	-2.2	-1.1	-2.4	-2
NM_004702.1 cyclin E2 (CCNE2)	NC	NC	-1.7	NC	-2.3	-4.3
NM_004049.1 BCL2-related protein A1 (BCL2A1)	NC	NC	-1.4	-1.2	-2.1	-3.5
D64137 KIP2 gene for Cdk-inhibitor p57KIP2	NC	2.2	4.3	2.3	4.5	4.5
NM_000076.1 cyclin-dependent kinase inhibitor 1C(p57Kip2)	NC	1.6	1.1	2.1	3.4	4.7
NM_030579.1 cytochrome b5	-1.4	-1.4	-6.9	NC	-1.6	-1.9
Cell signal transduction pathways						
NM_003954.1 MAP kinase kinase kinase 14	-1.1	-1.6	-2.3	-1.1	-2.7	-3.9
NM_002756.1 MAP kinase kinase 3 (MAP2K3)	-1.2	1.1	-1.7	NC	-2.3	-2.7
NM_002376.1 MAP regulating kinase 3 (MARK3)	-1.3	-1.7	-3.3	NC	-2.2	-3.1
NM_003010.1 MAP kinase kinase 4 (MAP2K4)	-1.3	-1.7	-4.1	-1.1	-2	-2.9
NM_006218.1 phosphoinositide-3-kinase	-1.4	-1.2	-2.1	NC	NC	NC
NM_002646.1 phosphoinositide-3-kinase	-3	-1.4	-2.4	NC	NC	NC
Transcription factor, Oncogenesis, Angiogenesis, Other						
NM_007111.1transcription factor Dp-1 (TFDP1)	-1.2	-1.3	-3.8	NC	-2.1	-3.7
U62296.1 transcription factor NF-YC	-1.2	-1.5	-3.4	NC	-2	-2.1
BE966878 eukaryotic protein synthesis initiation factor 4	NC	NC	NC	-1.1	-1.8	-2.1
NM_001755.1core-binding factor, beta subunit (CBFB)	-1.2	-1.6	-2.9	NC	-2	-2.1
NM_006850.1 suppression of tumorigenicity 16 (ST16)	2.2	3.6	2.4	1.2	1.7	2.4
AI812030 thrombospondin 1	NC	-1.9	-4.7	-1	-3	-4.3
BE999967 thrombospondin 1	-1	-1.4	-3.9	-1.2	-3.3	-5
BE620457 neuropilin 1	-1.6	-1.9	-8.2	NC	NC	NC
M92934.1connective tissue growth factor	NC	NC	NC	-1.9	-14.7	-19.8

*: Positive value, Increase; **: No Change; ***: Negative value, Decrease.

Conclusion: The task-1 has been fully completed, which was initiated during the submission and successful funding of this proposal.

Task-2. Our task-2 was to investigate the relationship between Akt and NF-κB, the effects of I3C and DIM on Akt and NF-κB pathways, and the effects of I3C and DIM on apoptosis through regulation of Akt and NF-κB pathways.

1. I3C and DIM significantly inhibited Akt activation.

By Western Blot analysis, we have found that I3C and DIM significantly inhibited Akt activity in PC-3 prostate cancer cells (Figure 11 and 12). The level of phosphorylated Akt was decreased upon I3C or DIM treatment. However, the level of Akt remained unchanged after I3C or DIM treatment.

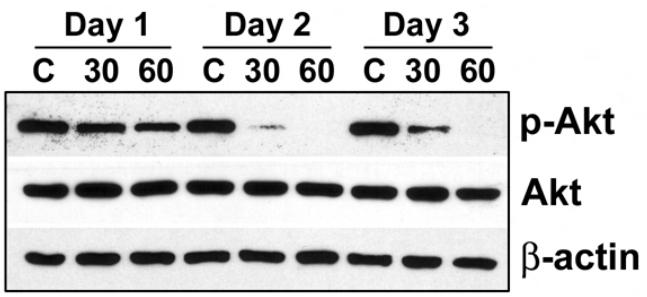


Figure 11: *Western Blot analysis showed that I3C treatment for 24-72 hours significantly inhibited the phosphorylation of Akt in PC-3 prostate cancer cells. Note that there was no change in the level of total Akt in I3C-treated PC-3 cells. (C: control; 30, 60: 30, 60 μM I3C)*

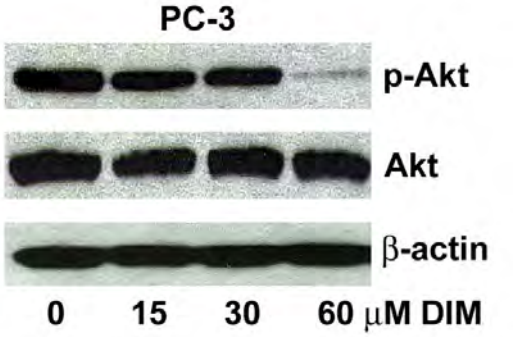


Figure 12: *Western Blot analysis showing that DIM treatment for 72 hours significantly inhibits the phosphorylation of Akt in PC-3 prostate cancer cells.*

2. The crosstalk between Akt and NF-κB pathways and the interruption of the crosstalk by I3C and DIM.

To investigate the relationship between Akt and NF-κB, we have conducted transfection experiments with Akt (wild, mutant, or constitutively activated) expression vectors and NF-κB-Luc vector that contains NF-κB binding and reporter sequences, and measured the activity of luciferase as well as NF-κB DNA binding activity in transfected prostate cancer cells. We have also detected the effects of I3C and DIM on Akt and NF-κB in transfected and un-transfected prostate cancer cells.

1). The effects of DIM.

We have found that Akt transfection up-regulated the activity of NF-κB as demonstrated by luciferase assay (Figure 13) and EMSA (Figure 14). We also found that DIM inhibited the activation of NF-κB induced by Akt transfection (Figure 13 and 14), suggesting that the inhibition of NF-κB by DIM is partly mediated by Akt pathway.

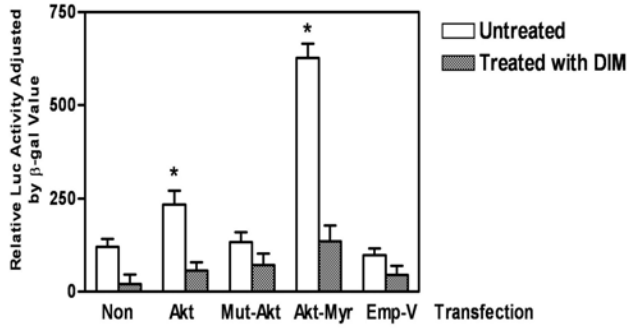


Figure 13: *Luciferase activity in transfected PC-3 cells with or without DIM treatment. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: p<0.05; n=2)*

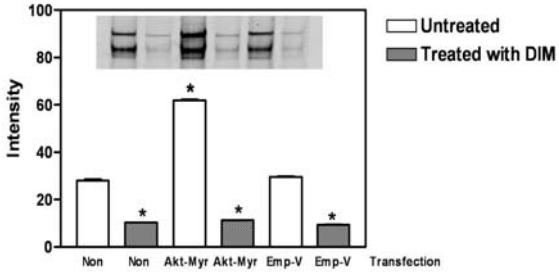


Figure 14: *EMSA and densitometric analysis of NF-κB DNA-binding activity in transfected PC-3 cells with or without DIM treatment. (Non: no transfection; Akt-Myr: transfected with constitutively activated Akt; Emp-V: transfected with empty vector; *: p<0.05; n=2)*

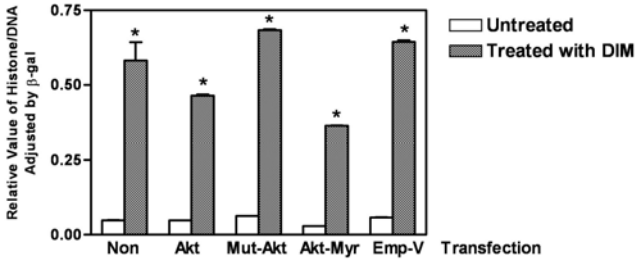


Figure 15: *Induction of apoptosis in DIM treated PC-3 cells tested by ELISA. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: p<0.05; n=2)*

We have also investigated the pro-apoptotic effect of DIM in PC-3 prostate cancer cells and Akt transfected PC-3 cells. We found that DIM induced apoptosis in both parental and Akt transfected PC-3 cancer cells (Figure 15). However, Akt transfection showed inhibitory effect on the apoptosis induced by DIM, suggesting that the pro-apoptotic effect of DIM is mediated by Akt and NF-κB pathways.

The results of our study described above have been published in the journal "Frontiers in Bioscience" [(13); please see Appendix 6]. The summary of our results in the form of an abstract is presented below.

Abstract: Prostate cancer is the second leading cause of cancer related deaths in men in the United States. I3C and its *in vivo* dimeric product, DIM, have been found to inhibit the growth of prostate cancer cells. However, the molecular mechanism(s) by which DIM elicits its effects on prostate cancer cells has not been fully elucidated. We have previously shown that I3C induces apoptosis and inhibits the activation of NF-κB pathway, which could be mediated via Akt signaling pathway. In this study, we investigated whether there is any cross-talk between Akt and NF-κB during DIM-induced apoptosis in PC-3 prostate cancer cells. We found that DIM inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells but not in non-tumorigenic CRL2221 human prostate epithelial cells. DIM also inhibited EGFR expression, PI3K kinase activity, and Akt activation, and abrogated the EGF-induced activation of PI3K in prostate cancer cells. NF-κB DNA-binding analysis and transfection studies with Akt cDNA constructs revealed that Akt transfection resulted in the induction of NF-κB activity and this was inhibited by DIM treatment. DIM treatment also showed significant induction of apoptosis in non-transfected cells compared to Akt and Akt-Myr transfected prostate cancer cells. From these results, we conclude that the inhibition of Akt and NF-κB activity and their cross-talk is a novel

mechanism by which DIM inhibits cell growth and induces apoptotic processes in prostate cancer cells but not in non-tumorigenic prostate epithelial cells.

2). The effects of I3C.

We have also investigated whether I3C have similar effects as DIM on Akt, NF-κB, and apoptotic pathways. We have conducted transfection experiments with Akt (wild, mutant, or constitutively activated) expression vectors and NF-κB-Luc vector that contains NF-κB binding and reporter sequences, and measured the activity of luciferase as well as NF-κB DNA binding activity in transfected PC-3 prostate cancer cells. We have detected the effects of I3C on Akt and NF-κB in transfected and un-transfected PC-3 prostate cancer cells. We found that Akt transfection up-regulated activity of NF-κB as demonstrated by luciferase assay (Figure 16) and EMSA (Figure 17). We also found that I3C inhibited the activation of NF-κB induced by Akt transfection (Figure 16 and 17), suggesting that the inhibition of NF-κB by I3C is partly mediated by Akt pathway.

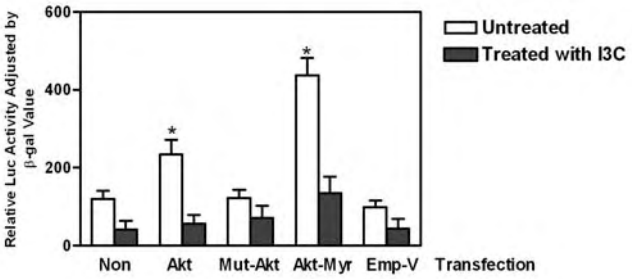


Figure 16: *Luciferase activity in transfected PC-3 cells with or without 60 μM I3C treatment. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: p<0.05; n=2)*

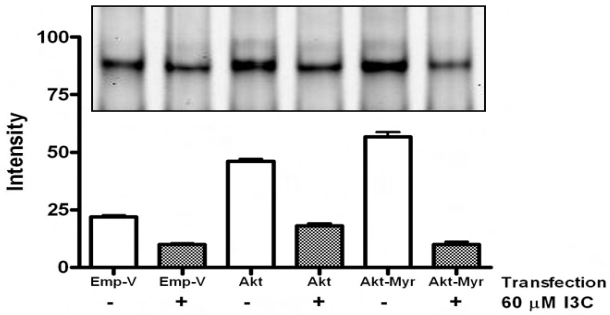


Figure 17: *EMSA and densitometric analysis of NF-κB DNA-binding activity in transfected PC-3 cells with or without I3C treatment. (Emp-V: transfected with empty vector; Akt: transfected with wild Akt; Akt-Myr: transfected with constitutively activated Akt.)*

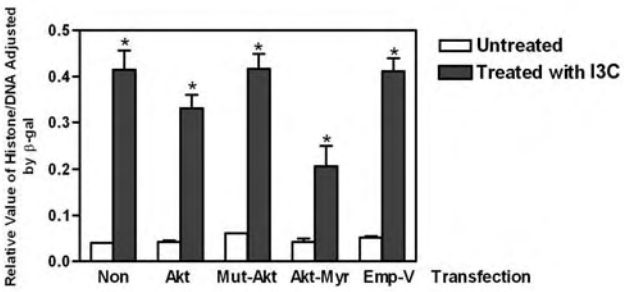


Figure 18: *Induction of apoptosis in 60 μM I3C treated PC-3 cells tested by ELISA. (Non: no transfection; Akt: transfected with wild Akt; Mut-Akt: transfected with mutant Akt; Akt-Myr: transfected with constitutively activated Akt; Emp-v: transfected with empty vector; *: p<0.05; n=2)*

We have also investigated the pro-apoptotic effect of I3C in PC-3 prostate cancer cells and Akt transfected PC-3 cells. We found that I3C induced apoptosis in both parental and Akt transfected PC-3 cancer cells (Figure 18). However, Akt transfection showed inhibitory effect on the apoptosis induced by I3C, suggesting that the pro-apoptotic effect of I3C is mediated by Akt and NF-κB pathways.

These results demonstrated that there is a crosstalk between Akt and NF-κB and both DIM and I3C could interrupt this crosstalk, resulting in the inhibition of cell growth and the induction of apoptosis in prostate cancer cells.

3. The inactivation of Akt and NF-κB pathways by DIM leads to the down-regulation of AR transactivation and PSA expression.

Because AR interacts with Akt and NF-κB pathways and plays important roles in the development and progression of prostate cancer, we have further investigated the effects of B-DIM, a specially formulated DIM with greater bioavailability, on Akt, NF-κB, and AR in hormone sensitive LNCaP and hormone insensitive C4-2B cells. These investigations are important for further elucidating the mechanistic role of NF-κB signaling pathway during I3C/DIM induced cell death. Therefore, we have conducted mechanistic experiments which were not included in task-2 but became necessary for continuation of our investigation in order to attract further funding from NIH.

1). Inhibition of AR and PSA expressions by DIM.

By Western Blot analysis, we found that B-DIM significantly inhibited the expression level of AR and PSA (Figure 19A and 19C) and the secretion of PSA (Figure 19B) in LNCaP prostate cancer cells in both time-dependent and dose-dependent manners. Furthermore, B-DIM abrogated the DHT-induced up-regulation of AR and PSA in LNCaP cells (Figure 19A and 19C).

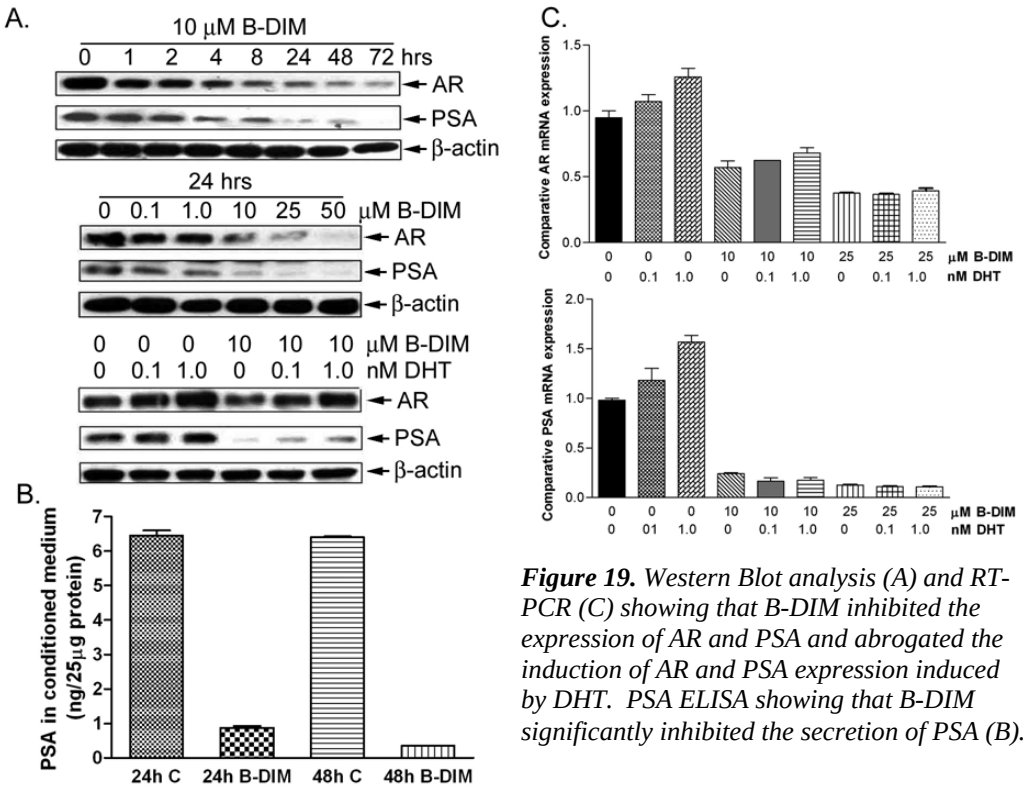


Figure 19. *Western Blot analysis (A) and RT-PCR (C) showing that B-DIM inhibited the expression of AR and PSA and abrogated the induction of AR and PSA expression induced by DHT. PSA ELISA showing that B-DIM significantly inhibited the secretion of PSA (B).*

2). Down-regulation of Akt by B-DIM leading to inhibition of NF-κB, AR, and PSA.

By Western Blot analysis, we found that B-DIM down-regulated the protein levels of p-Akt, nuclear NF-κB, AR, p-AR, and PSA in both LNCaP and C4-2B cells (Figure 20). To further investigate the relationship between Akt, NF-κB, AR, and PSA, and the effects of B-DIM on these molecules, we co-transfected Akt cDNA and NF-κB-Luc into LNCaP and C4-2B cells. We found that p-Akt, nuclear NF-κB, AR, p-AR(Ser213), and PSA were up-regulated after wild type

Akt and Myr Akt transfections in LNCaP and C4-2B cells. However, the up-regulations of p-Akt, nuclear NF-κB, AR, p-AR(Ser213), and PSA by Akt transfection were significantly abrogated by B-DIM treatment in both LNCaP and C4-2B cells (Figure 20).

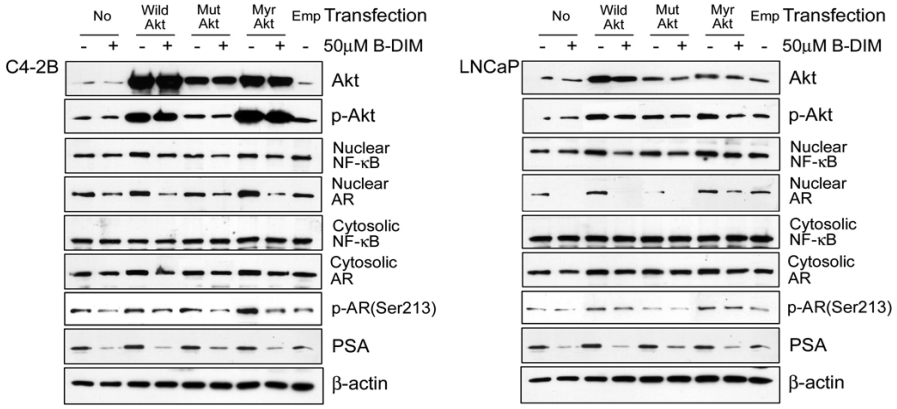


Figure 20. *B-DIM inhibited the expression of p-Akt, nuclear NF-κB, AR, and PSA in Akt transfected C4-2B and LNCaP cells. (Wild Akt: wild type Akt; Mut Akt: dominant negative Akt; Myr Akt: constitutively activated Akt; Emp: control empty vector.)*

By transfection and luciferase assay, we found that luciferase activity was significantly increased after co-transfection with NF-κB-Luc and wild type Akt or Myr Akt in both LNCaP and C4-2B cells (Figure 21A), suggesting the activation of NF-κB by Akt transfection. Moreover, B-DIM treatment significantly abrogated the up-regulation of luciferase activity caused by Akt transfection. Furthermore, we conducted EMSA to test NF-κB DNA binding activity in Akt transfected cells. The results showed that NF-κB DNA binding activity was significantly increased by wild type Akt and Myr Akt transfection and this was inhibited by B-DIM treatment (Figure 21B), which is consistent with the data from luciferase assay.

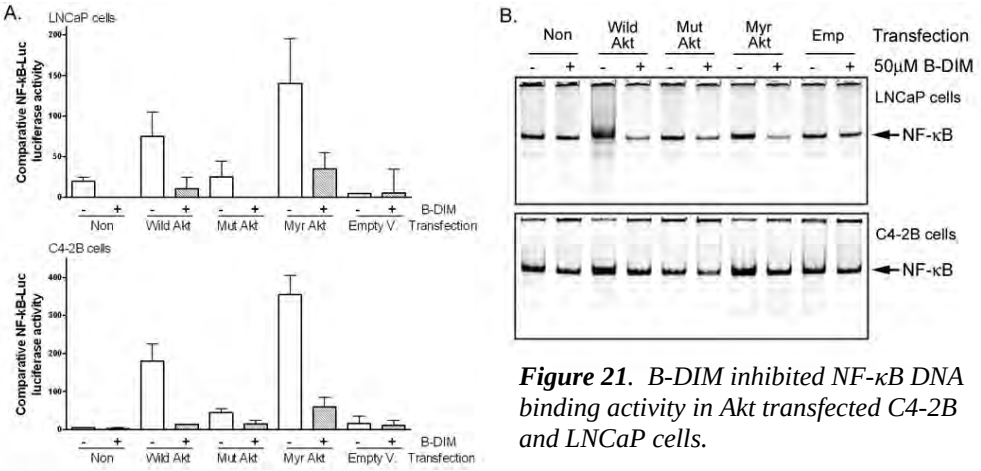


Figure 21. *B-DIM inhibited NF-κB DNA binding activity in Akt transfected C4-2B and LNCaP cells.*

Combined with the alteration in AR as observed by Akt transfection studies, our results suggest that there could be a crosstalk between p-Akt, NF-κB, and AR, and that the inhibition of Akt activation by B-DIM could lead to the down-regulation of NF-κB, AR, and PSA.

3). Inhibition of AR nuclear translocation by B-DIM.

By Western Blot analysis, we found that B-DIM reduced AR protein level in both the cytosol and the nuclear extracts (Figure 20). However, the AR protein level was much more down-regulated by B-DIM in the nucleus than in the cytosol, suggesting that B-DIM could inhibit AR nuclear translocation. Therefore, we conducted immunofluorescent staining and confocal imaging to examine the effect of B-DIM on AR nuclear translocation in LNCaP and

C4-2B prostate cancer cells. We found that both LNCaP and C4-2B cells treated with 25 μM B-DIM for 24 hours showed much less AR staining in the nucleus compared to control (Figure 22A and 22B). These results demonstrated that B-DIM significantly inhibited AR translocation into the nucleus and, in turn, down-regulated AR target genes including PSA, consistent with RT-PCR and Western Blot data showing down-regulation of PSA mRNA and protein levels after B-DIM treatment (Figure 19).

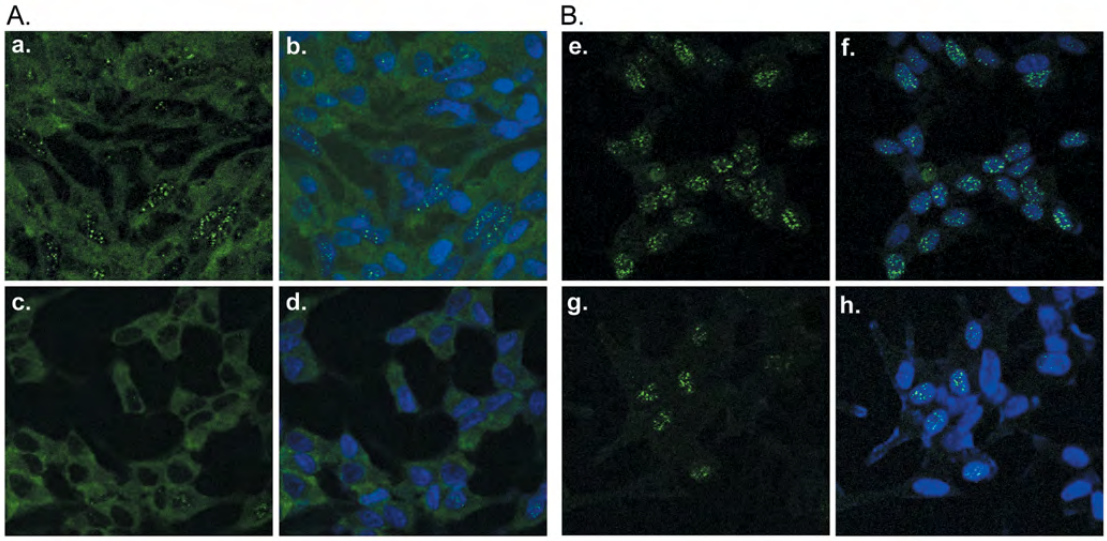


Figure 22. *B-DIM significantly inhibited AR nuclear translocation in LNCaP (A) and C4-2B (B) cells. (a and b: LNCaP cell control; c and d: LNCaP cells treated with 25 μM B-DIM; e and f: C4-2B cell control; g and f: C4-2B cells treated with 25 μM B-DIM.)*

There results suggest that down-regulation of AR and PSA by B-DIM could be mediated through Akt and NF-κB signaling. The above data has been presented in AACR annual meeting (14) and published in Cancer Research [(15); please see Appendix 7 and 9].

4. Inhibition of Akt and NF-κB by I3C and DIM leads to chemosensitization.

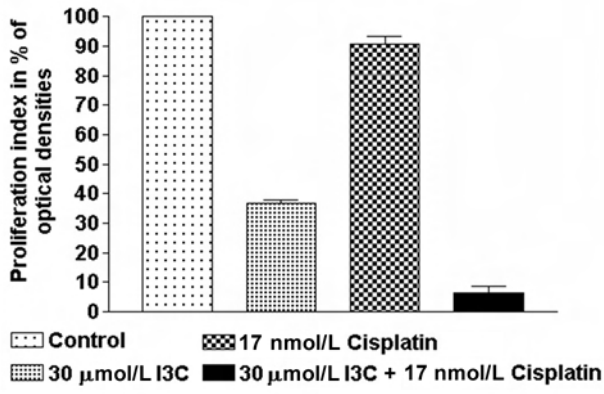


Figure 23. *Growth inhibition of PC-3 prostate cancer cells exposed to I3C for 24h and then cisplatin for 48 h. Combination treatment with I3C and cisplatin significantly inhibited the growth of PC-3 cells compared to monotreatment.*

The activation of Akt and NF-κB 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-κB or Akt may potentiate the anticancer effect of chemotherapeutic agents. We have found that I3C inhibited the activation of Akt and NF-κB and induced apoptosis, suggesting that I3C can sensitize cancer cells to apoptotic death induced by chemotherapeutic agents. By cell growth inhibition assay, we found that combination treatment with I3C and cisplatin significantly inhibited the growth of PC-3 prostate cancer cells compared to monotreatment (Figure 23). These results

suggest that I3C could potentiate the anticancer effect of chemotherapeutic agents through inactivation of Akt and NF-κB.

To investigate whether DIM also sensitizes prostate cancer cells to death induced by chemotherapeutic agents, we tested the effects of combination treatment with DIM and Taxotere on the growth of LNCaP, C4-2B, and, PC-3 prostate cancer cells. We found that the combination treatment with DIM and Taxotere exerted significantly more growth inhibitory effect on these cells (Figure 24). The results of our study have been presented in 96th AACR Annual Meeting (14). The summary of our results in the form of an abstract is presented below.

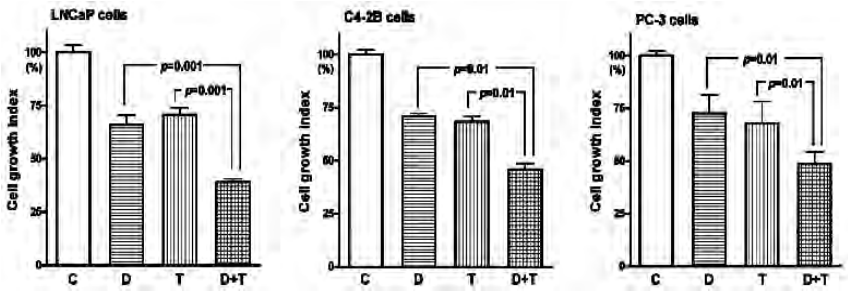


Figure 24. *Growth inhibition assay showed that combination treatment with DIM and Taxotere for 72h significantly inhibited the growth of LNCaP, C4-2B, and PC-3 cells compared to monotreatment.*

Abstract: Despite initial response to androgen deprivation, patients with prostate cancer usually develop hormone refractory metastasis and eventually succumb to the disease. Therefore, new therapeutic strategies are urgently needed to overcome drug resistance for the treatment of prostate cancer. Epidemiological studies have shown that the consumption of cruciferous vegetables is associated with reduced cancer risk. 3,3'-diindolymethane (DIM) is an acid condensation product of I3C, which is present in almost all members of the cruciferous vegetable family. DIM has been shown to down-regulate AR, suggesting the growth inhibitory effects of DIM on hormone related cancers. A formulated DIM (B-DIM, BioResponse) has been shown to exhibit approximately 50% higher bioavailability. However, no molecular studies have been reported to date to elucidate the effect of B-DIM on prostate cancer cells. To investigate the effects of B-DIM on prostate cancer cells, we treated hormone sensitive LNCaP cells and hormone insensitive PC-3 cells with 0 to 60 μM of B-DIM for one to three days. MTT, ELISA, and Western blot analysis were conducted to test cell growth inhibition, apoptosis, and the expression of AR and prostate specific antigen (PSA). Microarray analysis was also conducted to assess global alternations in gene expression profile. We found that B-DIM inhibited the growth of prostate cancer cells and induced apoptosis in a dose and time dependent manner. Microarray gene expression analysis showed that B-DIM regulated the expression of genes which are critical for the control of cell growth and apoptosis. Western blot analysis showed that B-DIM significantly inhibited AR and PSA protein expression in LNCaP prostate cancer cells. It has been recently reported that increases in androgen receptor mRNA and proteins are both necessary and sufficient to convert prostate cancer from a hormone-sensitive to a hormone refractory stage, suggesting that over-expression of AR is an important factor for acquired resistance of prostate cancer to hormone ablation therapy. Since our results showed decreased AR and PSA expression with concomitant inhibition of cell growth and induction of apoptosis by B-DIM, we suggest that the resistance acquired by prostate cancer cells to androgen ablation therapy could be reversed by B-DIM treatment. Moreover, we tested our hypothesis whether B-DIM could sensitize hormone insensitive PC-3 prostate cancer cells to Taxotere. We found that Taxotere (1 nM) in combination with 50 μM B-DIM exerted significantly more growth

inhibitory effects compared to Taxotere or B-DIM treatment alone. In conclusion, our results provide a solid foundation for devising novel therapeutic strategies for the treatment of hormone or chemo-resistant metastatic prostate cancer by including B-DIM, a non-toxic agent, with other therapeutic agents.

Our published review articles [(16-19); please see Appendix 2, 4, 5, and 8] are also attached to provide comprehensive results that we have obtained. These progresses are above and beyond what we proposed in our application.

Conclusion: The task-2 has been fully completed.

Task-3. Our task 3 was to determine whether treatment of prostate cancer cells with I3C and DIM will elicit responses that may determine causal association between the inactivation of Akt and NF-κB with the induction of apoptosis. We have conducted transfection experiments using IKKα, IKKβ, MEKK, and NIK cDNA plasmids. We have measured the levels of IKKα, IKKβ, MEKK, NIK, IκBα, phosphor-IκBα, and NF-κB after transfections with these plasmids and I3C/DIM treatments. To extent our study, we have also investigated the consequent events after the inhibition of Akt and NF-κB by DIM. Our findings from these experiments are presented below.

1. Regulation of NF-κB related molecules by I3C and DIM.

1). IKKβ transfection.

In human cells, NF-κB is sequestered in the cytoplasm through tight association with its inhibitory protein, IκBα. The activation of NF-κB occurs through site-specific phosphorylation and ubiquitination of IκBα by IκB kinase β (IKKβ). IκBα is subsequently degraded by the 26S proteasome. NF-κB becomes free from IκBα and translocates into nucleus for binding to NF-κB-specific DNA-binding sites, regulating target gene transcription (Figure 25). Because IKKβ is the important molecule which regulates the activity of NF-κB, we have investigated the effects of I3C and DIM on IKK-NF-κB pathway by transfection of

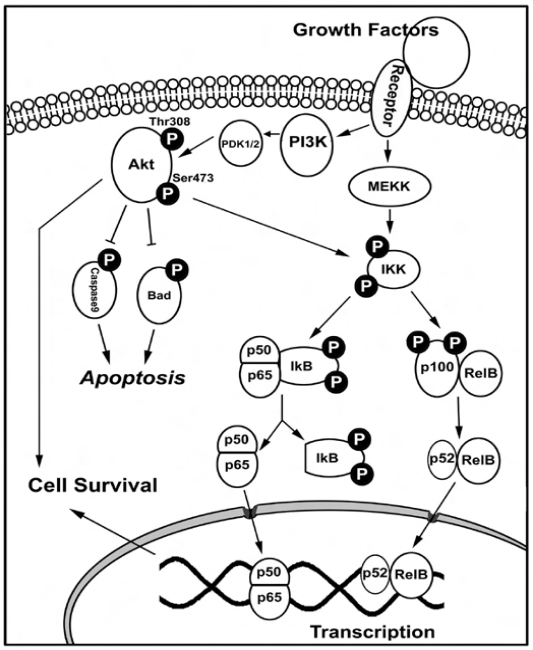


Figure 25. *Regulation of NF-κB and Akt signaling pathways.*

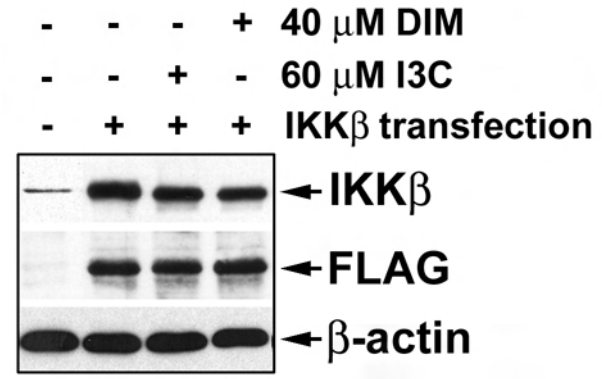


Figure 26. *Western Blot analysis showed that expression of IKKβ was significantly up-regulated with IKKβ cDNA transfection and was down-regulated by I3C and DIM treatment.*

IKKβ into prostate cancer cells.

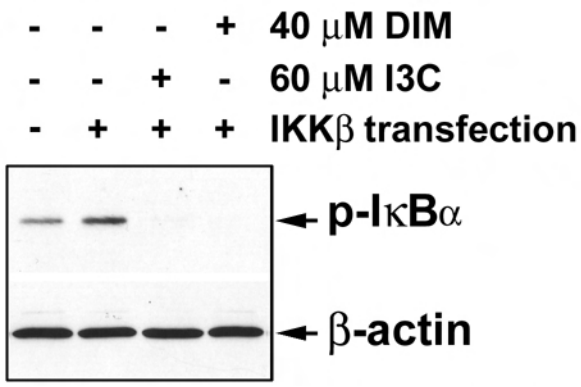


Figure 27. *Western Blot analysis showed that expression of phospho-IκBα was up-regulated with IKKβ cDNA transfection and was significantly down-regulated by I3C and DIM treatment.*

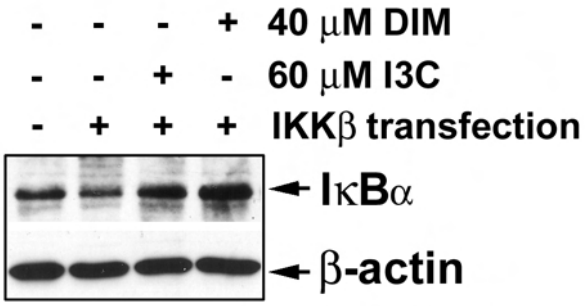


Figure 28. *Western Blot analysis showed that expression of IκBα was down-regulated with IKKβ cDNA transfection and was up-regulated by I3C and DIM treatment.*

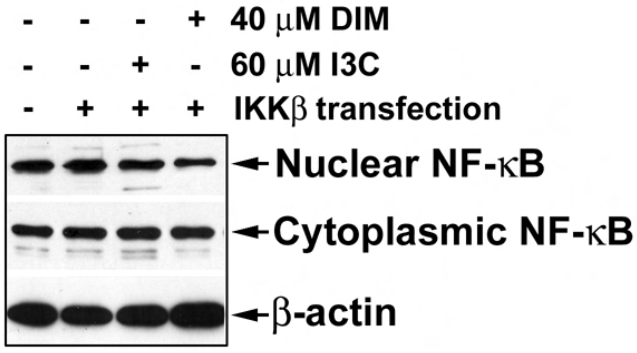


Figure 29. *Western Blot analysis showed that expression of nuclear NF-κB was up-regulated with IKKβ cDNA transfection and was down-regulated by I3C and DIM treatment. The level of cytoplasmic NF-κB remained unchanged.*

We have transfected IKKβ cDNA into PC-3 prostate cancer cells. The IKKβ transfected cells were treated with or without 60 μM I3C or 40 μM DIM. We found that the expression of IKKβ was significantly up-regulated with IKKβ cDNA transfection (Figure 26). The transfection efficacy was equal in all transfection samples because the equal expression of FLAG was observed. We also found that I3C or DIM inhibited the expression of IKKβ in PC-3 prostate cancer cells (Figure 26).

Since we observed the down-regulation of IKKβ by I3C and DIM, we tested the levels of phospho-IκBα protein, which has been known to be produced from phosphorylation of IκBα by IKKβ. We found more phospho-IκBα protein in IKKβ transfected PC-3 cells compared to parental cells. Importantly, significant down-regulation of phosphorylated IκBα was observed in IKKβ transfected cells treated with I3C or DIM, suggesting that the inhibition of IκBα phosphorylation by I3C or DIM may be mediated by the down-regulation of IKKβ (Figure 27).

We also tested the level of IκBα protein. We found that the transfection of IKKβ caused a slight decrease in the level of IκBα. However, I3C and DIM treatment increased the level of IκBα protein, suggesting the lower degree of IκBα phosphorylation after I3C and DIM treatment (Figure 28), and these results are consistent with results presented in Figure 28.

Because we observed the inhibitory effects of I3C and DIM on the IκBα phosphorylation that activates NF-κB, we measured the levels of NF-κB protein in cytoplasmic and nuclear extracts, respectively. We found that the level of cytoplasmic NF-κB remained unchanged upon I3C or

DIM treatment. However, the level of nuclear NF-κB was significantly decreased after I3C or DIM treatment, suggesting that I3C and DIM inhibited the nuclear localization of NF-κB (Figure 29).

We also tested the DNA binding activity of nuclear NF-κB by EMSA. We found that I3C and DIM inhibited the DNA binding activity of nuclear NF-κB in a time-dependent manner in IKKβ transfected PC-3 cells (Figure 30), consistent with our Western Blot data.

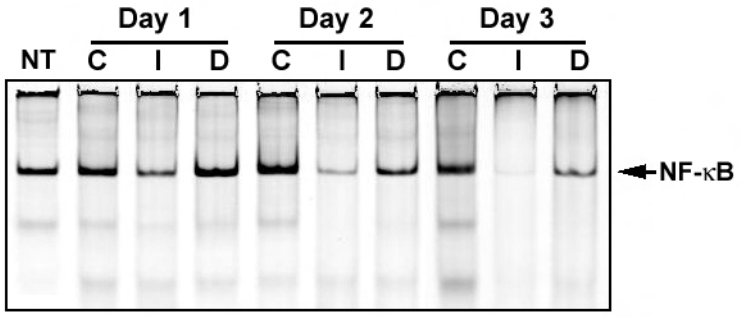


Figure 30. *EMSA showed that the DNA binding activity of NF-κB was down-regulated by I3C and DIM treatment in IKKβ transfected cells (NT: no transfection; C: control; I: treated with 60 μM I3C; D: treated with 40 μM DIM).*

These results suggest that I3C and DIM may inhibit the expression of IKKβ and the subsequent phosphorylation of IκBα, resulting the inhibition of nuclear localization and activation of NF-κB. These effects of I3C and DIM may be causally related to the inhibition of cancer cell growth and the induction of apoptosis as observed in our earlier results.

2). IKKα transfection.

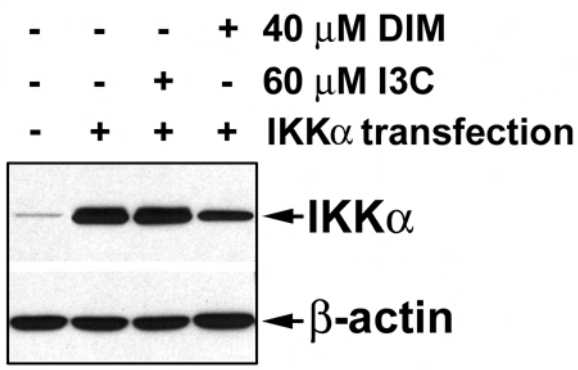


Figure 31. *Western Blot analysis showed that expression of IKKα was significantly up-regulated with IKKα cDNA transfection and was down-regulated by DIM treatment but I3C appears to be ineffective.*

The activation of NF-κB also occurs through phosphorylation of p100 by IKKα, leading to the processing of p100 into small form (p52). This process allows NF-κB (p52-RelB) to transfer into the nucleus for binding to NF-κB-specific DNA-binding sites and, in turn, regulating gene transcription (Figure 25). Because of the importance of IKKα in the NF-κB pathway, we have also transfected IKKα cDNA into PC-3 prostate cancers. The IKKα transfected cells were treated with or without 60 μM I3C or 40 μM DIM. We found that the expression of IKKα was significantly up-regulated with IKKα cDNA transfection. We also found that DIM inhibited the expression of IKKα in PC-3 prostate cancer cells, but no significant effect was observed with I3C (Figure 31).

3). MEKK transfection.

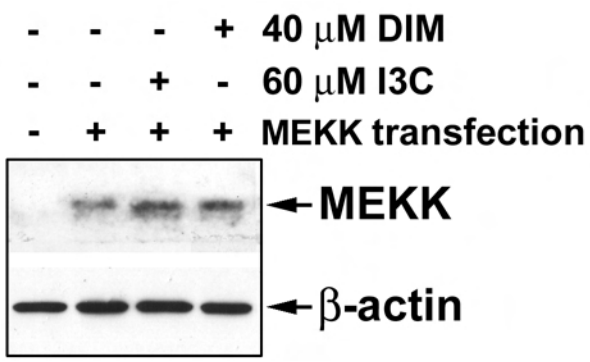


Figure 32. *Western Blot analysis showed that expression of MEKK was up-regulated with MEKK cDNA transfection. No significant alteration in the expression of MEKK was observed by I3C and DIM treatment in MEKK transfected PC-3 cells.*

It has been known that IKK could be phosphorylated and activated by an upstream kinase, mitogen activated kinase kinase 1 (MEKK1). Therefore, MEKK is another important molecule which regulates the activity of NF-κB (Figure 25). Hence, we have transfected MEKK cDNA into PC-3 prostate cancer cells. The MEKK transfected cells were treated with or without 60 μM I3C or 40 μM DIM. We found that the expression of MEKK was up-regulated with MEKK cDNA transfection. We did not observe significant alteration in the expression of MEKK in I3C or DIM treated PC-3 prostate cancer cells (Figure 32), suggesting that MEKK is not a target of I3C or DIM.

We have also tested the effects of I3C and DIM on MEK and NIK. However, we did not observe significant alteration in the level of MEK and NIK in I3C and DIM treated PC-3 cells transfected with MEK and NIK (data not shown), suggesting that MEK and NIK may not be targets of I3C and DIM.

2. Regulation of Akt related molecules by DIM.

It has been known that FOXO3a and GSK-3β, two targets of activated Akt, interact with β-catenin, regulating cell proliferation and apoptotic death. More importantly, FOXO3a, GSK-3β, and β-catenin all are AR coregulators and regulate the activity of AR, mediating the development and progression of prostate cancers. In addition, activated Akt has also been found to phosphorylate and transactivate AR. These findings demonstrate the importance of FOXO3a, GSK-3β, and β-catenin in Akt regulated cell survival and apoptotic death. Therefore, to extent our research on the molecular role of I3C/DIM, we have further investigated the effects of B-DIM on Akt related molecules, FOXO3a, GSK-3β, and β-catenin. These experiments were not included in task-3 but become necessary for continuation of our investigation. Following is our findings, which document our progress above and beyond what we proposed.

1). Inhibition of FOXO3a phosphorylation by B-DIM.

We tested the effects of B-DIM on FOXO3a, one of the main downstream target genes of Akt. We observed that phosphorylation of Akt and FOXO3a was upregulated by IGF-1 and down-regulated by LY294002 in prostate cancer cells as expected (Figure 33A and 33C). We found that B-DIM significantly inhibited the phosphorylation of FOXO3a in C4-2B and LNCaP cells (Figure 33B). Moreover, B-DIM abrogated the up-regulation of Akt and FOXO3a phosphorylation induced by IGF-1 (Figure 33). Furthermore, we found that B-DIM significantly inhibited the phosphorylation of FOXO3a at Thr32 and Ser253 and decreased the ratio of p-FOXO3a over FOXO3a in both the cytoplasm and nucleus of prostate cancer cells (Figure 33C), suggesting that B-DIM could help to retain a greater amount of FOXO3a in activated form to inhibit cancer cell growth. By immunoprecipitation, we found that B-DIM significantly inhibited the formation of FOXO3a and Akt complex in the cytosol and nucleus of prostate

cancer cells (Fig. 33D), consistent with Akt inactivation and subsequently decreased FOXO3a phosphorylation by B-DIM treatment. We also found that B-DIM significantly decreased the formation of FOXO3a and AR complex which could inhibit FOXO3a activity in both cytoplasmic and nuclear components (Fig. 33D), suggesting that greater level of active FOXO3a proteins will exert their anti-proliferative and pro-apoptotic effects.

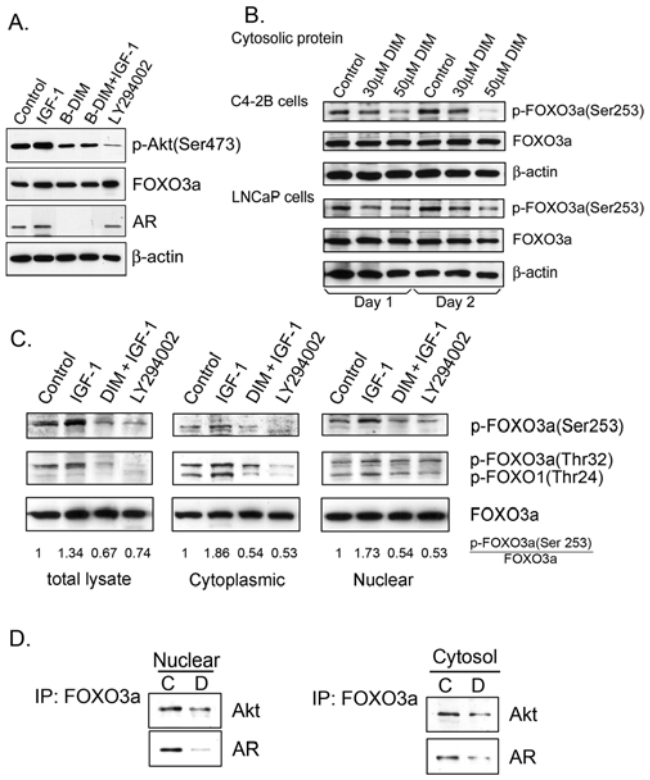


Figure 33*. B-DIM inhibited phosphorylation of Akt and FOXO3a. A: C4-2B cells were treated with 100 ng/ml IGF-1, 20 μM LY294002, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 hours. Total lysate from each sample was subjected to Western Blot analysis. B: LNCaP and C4-2B cells were treated with 50 μM B-DIM for 24 and 48 hours. Cytoplasmic lysate from each sample was subjected to Western Blot analysis. C: C4-2B cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM plus 100 ng/ml IGF-1, or 20 μM LY294002 for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to Western Blot analysis. The ratio of p-FOXO3a over FOXO3a was calculated by standardizing the ratios of each control to the unit value. D: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Cytoplasmic and nuclear lysates from each sample were subjected to immunoprecipitation and Western Blot analysis (C: control; D: B-DIM treatment).*

2). Increased GSK-3β binding to β-Catenin and induction of β-Catenin phosphorylation by B-DIM.

By Western Blot analysis, we found that B-DIM significantly decreased the level of β-catenin protein in the cytosol and nucleus of C4-2B cells (Figure 34A and 34B). More importantly, we found that B-DIM enhanced the phosphorylation of β-catenin and increased the ratio of p-β-catenin over β-catenin in the nucleus of C4-2B cells (Figure 34B). By immunoprecipitation, we discovered that GSK-3β binding to β-catenin was increased upon B-DIM treatment (Figure 34C), consistent with the increase in the phosphorylation of β-catenin. We also found that B-DIM inhibited FOXO3a binding to β-catenin (Figure 34C). In LNCaP cells, we also observed similar effects of B-DIM on the down-regulation of β-catenin level and up-regulation of β-catenin phosphorylation. These results suggest that B-DIM treatment leads to a

greater level of inactivated β-catenin and less of activated β-catenin in prostate cancer cells, causing a decrease in the activated β-catenin in the nucleus of prostate cancer cells.

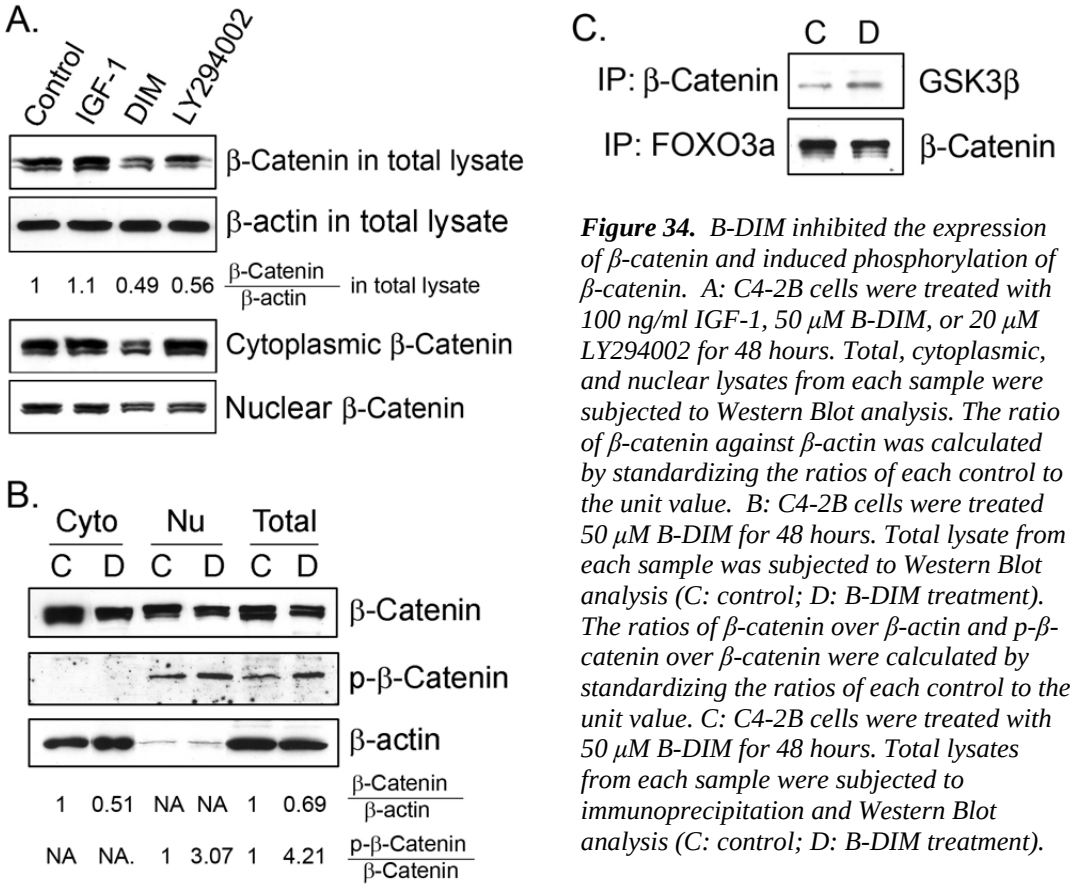


Figure 34. *B-DIM inhibited the expression of β-catenin and induced phosphorylation of β-catenin. A: C4-2B cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM, or 20 μM LY294002 for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to Western Blot analysis. The ratio of β-catenin against β-actin was calculated by standardizing the ratios of each control to the unit value. B: C4-2B cells were treated 50 μM B-DIM for 48 hours. Total lysate from each sample was subjected to Western Blot analysis (C: control; D: B-DIM treatment). The ratios of β-catenin over β-actin and p-β-catenin over β-catenin were calculated by standardizing the ratios of each control to the unit value. C: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Total lysates from each sample were subjected to immunoprecipitation and Western Blot analysis (C: control; D: B-DIM treatment).*

3). Inhibition of β-Catenin nuclear localization by B-DIM.

We observed lower level of β-catenin protein in the nucleus after B-DIM treatment (Figure 35A and 35B). By immunofluorescent staining, we found that B-DIM significantly inhibited the nuclear localization of β-catenin; however, no significant change in the nuclear localization of FOXO3a was observed upon B-DIM treatment (Figure 35A). To confirm these results, we also tested the expression of karyopherin, one of the nuclear importins, and the interaction of β-catenin with karyopherin in B-DIM treated and un-treated prostate cancer cells. We found that B-DIM significantly decreased the level of karyopherin protein in cytoplasm and nucleus of C4-2B cells (Figure 35B). More importantly, we found that B-DIM significantly inhibited karyopherin binding to β-catenin, consistent with the data from immunofluorescent staining, demonstrating the inhibitory effects of B-DIM on the nuclear translocation of β-catenin. However, we did not observe such a significant inhibitory effect of B-DIM on the binding of karyopherin to FOXO3a (Figure 35C). We also observed similar effects of B-DIM on the relocation of β-catenin in LNCaP cells. Since FOXO3a as a transcription factor could regulate the transcription of its downstream genes including AR and $p27^{KIP1}$, we tested the effects of B-DIM on the transcription activity of AR and $p27^{KIP1}$ regulated by FOXO3a.

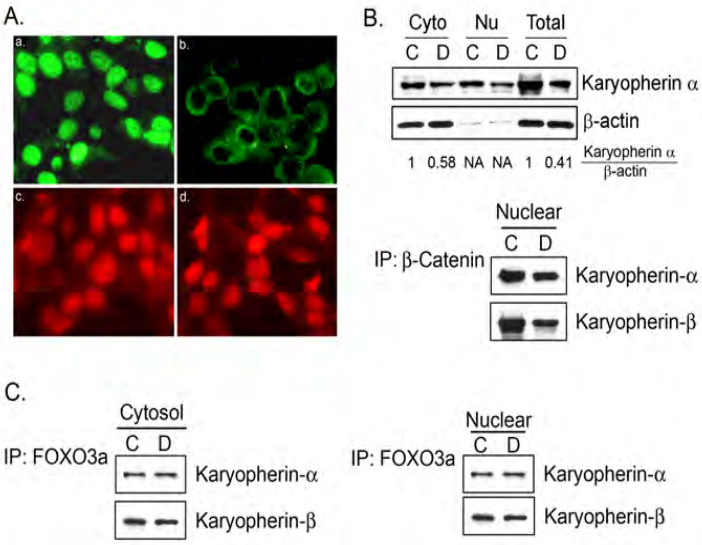
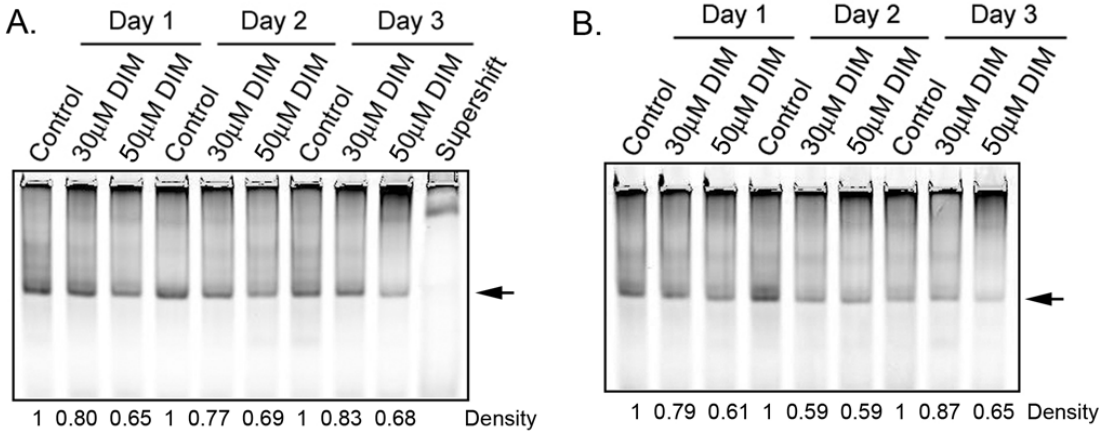


Figure 35. *B-DIM inhibited the nuclear translocation of β-catenin but did not alter FOXO3a nuclear translocation. A: C4-2B cells were treated with 50 μM B-DIM for 48 hours. The samples were subjected to immunofluorescent staining using anti-β-catenin (a and b) and anti-FOXO3a (c and d) antibodies. (a and c: control; b and d: B-DIM treatment). B, C, and D: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to immunoprecipitation and/or Western Blot analysis (C: control; D: B-DIM treatment; Cyto: cytoplasmic; Nu: nuclear). The ratio of Karyopherin α over β-actin was calculated by standardizing the ratios of each control to the unit value.*

4). Inhibition of FOXO3a mediated AR transcription by B-DIM.

By EMSA, we found that B-DIM inhibited the activity of FOXO3a protein binding to AR promoter DNA in C4-2B and LNCaP cells (Figure 36A and 36B), suggesting the inhibitory effect of B-DIM on the FOXO3a induced transcription of AR. We also conducted ChIP assay with conventional and real-time PCR to confirm this effect of B-DIM in a live cell environment. We found that B-DIM treatment decreased FOXO3a protein binding to AR promoter *in vivo* in C4-2B (Figure 36C and 36D) and LNCaP prostate cancer cells. By Western Blot analysis, we indeed observed the down-regulation of AR expression by B-DIM treatment (Figure 33A), consistent with the results from EMSA and ChIP assay.



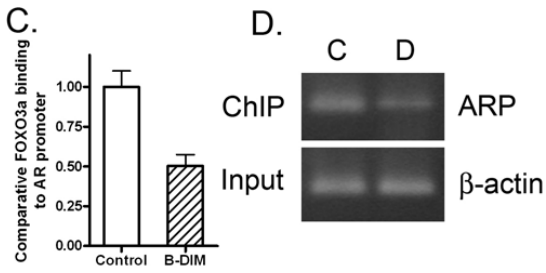


Figure 36. *B-DIM inhibited FOXO3a binding to the promoter of AR. Nuclear proteins from C4-2B (A) and LNCaP (B) cells were subjected to EMSA using AR oligonucleotide and ChIP assay using real-time PCR (C) or conventional PCR (D) (ARP: AR promoter primers; C: control; D: B-DIM treatment; Arrow indicating the specific DNA binding. The density of each signal was calculated by standardizing each control to the unit value).*

5). Stimulation of FOXO3a mediated p27^{KIP1} expression by B-DIM.

In contrast with AR, using EMSA we found that B-DIM significantly increased the activity of FOXO3a protein binding to p27^{KIP1} promoter DNA in C4-2B cells (Figure 37A). ChIP assay confirmed EMSA data, showing that B-DIM significantly enhanced FOXO3a protein binding to p27^{KIP1} promoter *in vivo* in prostate cancer cells (Figure 37B and 37C). Furthermore, we found increased level of p27^{KIP1} protein after B-DIM treatment (Figure 37D), consistent with the data from EMSA and ChIP assay. We also observed similar results in LNCaP cells. Since FOXO3a, GSK-3β, and β-catenin all are important factors which regulate cell survival and apoptotic cell death, we tested the effects of B-DIM on cell proliferation and apoptosis.

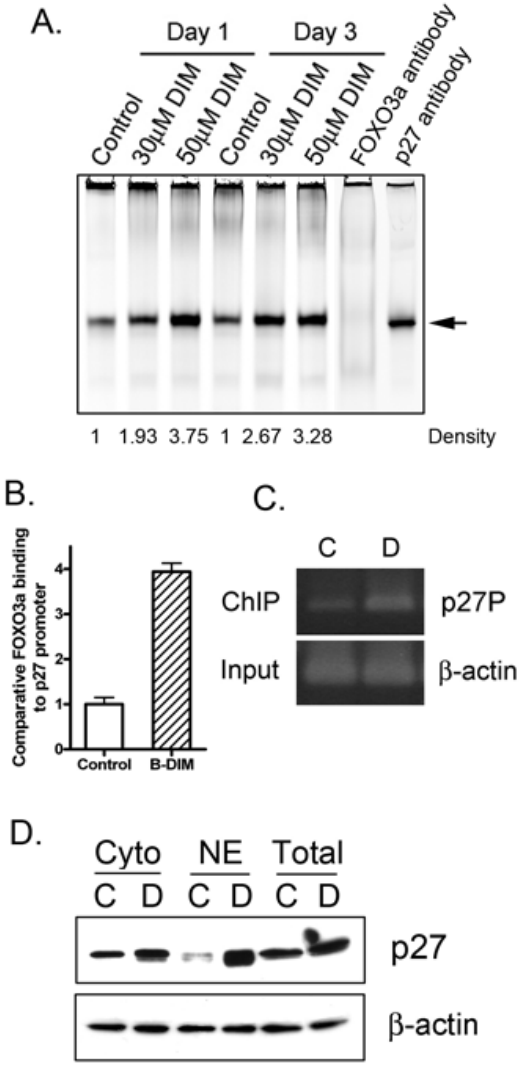


Figure 37. *B-DIM up-regulated FOXO3a binding to the promoter of p27KIP1. A: C4-2B cells were treated with 30 or 50 μM B-DIM for 24 and 72 hours. Nuclear proteins from each sample were subjected to EMSA using p27KIP1 oligonucleotide. (Arrow indicating the specific DNA binding. The density of each signal was calculated by standardizing each control to the unit value). B and C: C4-2B cells were treated with50 μM B-DIM. The samples were then subjected to ChIP assay using real-time PCR (B; bars: average±SD; n=3) or conventional PCR (C; p27P: using p27KIP1 promoter primers; C: control; D: B-DIM treatment). D: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to Western Blot analysis (C: control; D: B-DIM treatment; Cyto: cytoplasmic; NE: nuclear extract).*

6). Inhibition of cell proliferation and induction of apoptosis by B-DIM through regulation of Akt/FOXO3a/β-Catenin/GSK-3β/AR signaling.

By MTT assay, we found that IGF-1, which activates Akt, promoted cell proliferation in LNCaP and C4-2B cells (Figure 38A and 38B). Importantly, B-DIM inhibited cell proliferation and abrogated IGF-1 induced cell proliferation (Figure 38A and 38B). By apoptosis assay, we observed that IGF-1 protected cancer cells from apoptosis (Figure 38C and 38D). However, B-

DIM induced apoptosis and abrogated IGF-1 mediated protection of LNCaP and C4-2B cells from apoptosis. These results are consistent with our observation on the alterations of Akt and FOXO3a caused by IGF-1 or B-DIM treatment (Figure 33A and 33C).

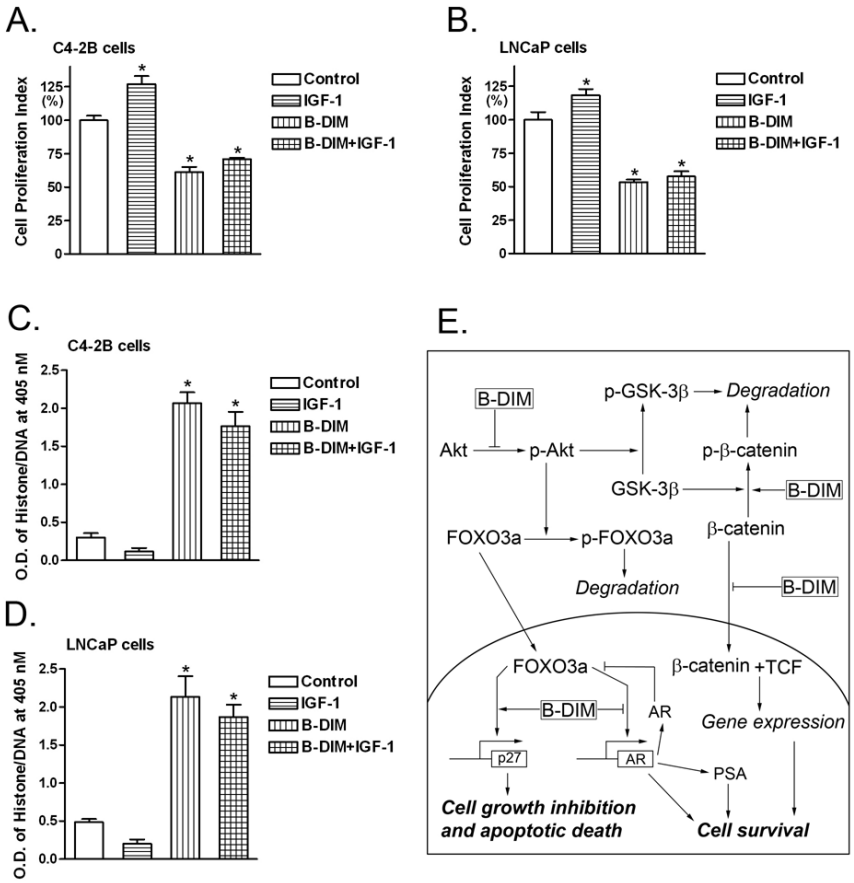


Figure 38. *A, B, C, and D: C4-2B and LNCaP cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 hours. Cell proliferation was tested by MTT assay (A and B). Apoptotic cell death was tested by Cell Death Detection ELISA (C and D). *: p<0.05, compared to control; n=3). E: Schematic representation of molecular effect of B-DIM on Akt/FOXO3a/GSK-3β/β-catenin/AR signaling.*

From these data, we conclude that the inhibition of cell proliferation and induction of apoptosis by B-DIM is partly mediated by the deregulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling pathways (Figure 38E). The above data has been submitted to JBC and will be presented in AACR annual meeting (please see Appendix 13 and 14).

3. Inactivation of NF-κB by B-DIM leads to the inhibition of angiogenesis and invasion mediated by NF-κB downstream genes.

To extent our research on NF-κB, we have investigated molecular effects of B-DIM on NF-κB downstream genes including VEGF, uPA, and MMP-9, which are critically involved in the induction of angiogenesis and invasion. These experiments were not included in task-3 but become necessary for continuation of our investigation, similar to those indicated earlier. Following is our findings.

1). B-DIM down-regulated VEGF at the level of transcription in LNCaP cells.

VEGF121, VEGF165, and VEGF189 are the major forms secreted by most cell types. We have conducted real time RT-PCR using isoform-specific VEGF primers and probes and tested the relative distribution of mRNA levels of major isoforms in LNCaP and C4-2B cells, respectively. We found that VEGF121 is the most abundant in prostate cancer cell line LNCaP and C4-2B cells, and VEGF165 is the lowest (Figure 39A). Importantly, we found that total VEGF and VEGF121 was significantly reduced by B-DIM treatment (Figure 39B). Promoter assay showed that B-DIM treatment induced significant decrease of luciferase activity in LNCaP cells (Figure 39C), suggesting that B-DIM down-regulated the transcription of VEGF.

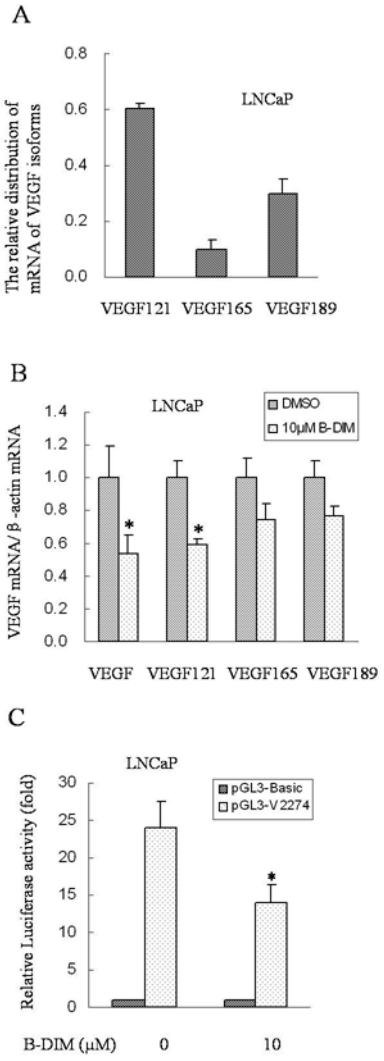


Figure 39. *The effects of B-DIM on VEGF mRNA and promoter activity and the relative distribution of mRNA levels of VEGF isoforms. (A) The relative distribution of mRNA levels of VEGF isoforms in LNCaP and C4-2B cells, respectively. (B) LNCaP and C4-2B cells were treated with B-DIM or DMSO as control. The total RNA was isolated and real time RT-PCR was performed for total VEGF, VEGF121, VEGF165, and VEGF189. (*, $p < 0.05$, $n = 4$). (C) pGL3-basic luciferase reporter or pGL3-2274 containing the VEGF promoter were cotransfected with cytomegalovirus-β-galactosidase plasmid into LNCaP and C4-2B cells. After 24h of transfection, the cells were treated with 10 μmol/L of B-DIM or DMSO as vehicle control for another 24h. Luciferase activity was then determined. Relative fold changes in luciferase activity was calculated. (*, $p < 0.05$ compared to without B-DIM control, $n = 6$).*

2). B-DIM reduced cellular VEGF and the secretion of VEGF.

In order to determine the effect of B-DIM on secretion of VEGF, we have analyzed the levels of VEGF in conditioned media from LNCaP and C4-2B cells treated with B-DIM. As shown in Figure 40A and 40B, 1 and 10 μmol/L of B-DIM treatment markedly decreased the VEGF secretion from LNCaP and C4-2B cells compared to DMSO control. Western blot

analysis showed that B-DIM treatment reduced cellular VEGF in LNCaP cells but had no significant effect on cellular VEGF in C4-2B cells (Figure 40C and 40D). The band with 57 kDa molecular weight corresponded with the long intact isoforms of VEGF189 predominantly associated with cell surface and extracellular matrix.

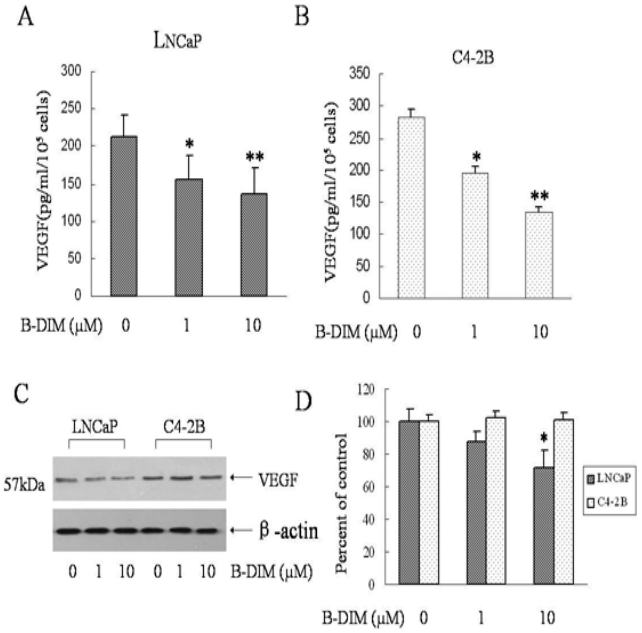


Figure 40. *The effects of B-DIM on the secretion and expression of cellular VEGF. (A) LNCaP and (B) C4-2B cells were grown in serum free medium for 24h. The cells were then treated with 1 and 10 μmol/L B-DIM or DMSO as vehicle control in 1% FBS for 24h. The culture media was collected and VEGF was assayed using an ELISA kit. Data were presented as mean ± SE; n = 6. *, p < 0.05, **, p < 0.01 compared to control. (C) Cell culture conditions were same as above. The cell lysates were subjected to gel electrophoresis and Western blot analysis using anti-VEGF antibody. (D) Quantitative analysis of VEGF expression in LNCaP and C4-2B cells was performed and relative density of bands normalized for β-actin Data were presented as mean ± SE; n = 3. *, p < 0.05 compared to control.*

3). B-DIM reduced bioavailability of VEGF through repressing MMP-9 expression.

Because MMP-9 is known to regulate bioavailability of ECM-binding VEGF via degrading the ECM, we have determined MMP-9 levels from conditioned media. As shown in Figure 41A and 41B, B-DIM significantly decreased the MMP-9 levels in culture supernatants compared with the control. Moreover, B-DIM dramatically repressed the MMP-9 protein expression (Figure 41A and 41B lower panel). To explore whether reduced MMP-9 expression by B-DIM was responsible for decreased bioavailability of VEGF, we assayed the VEGF levels

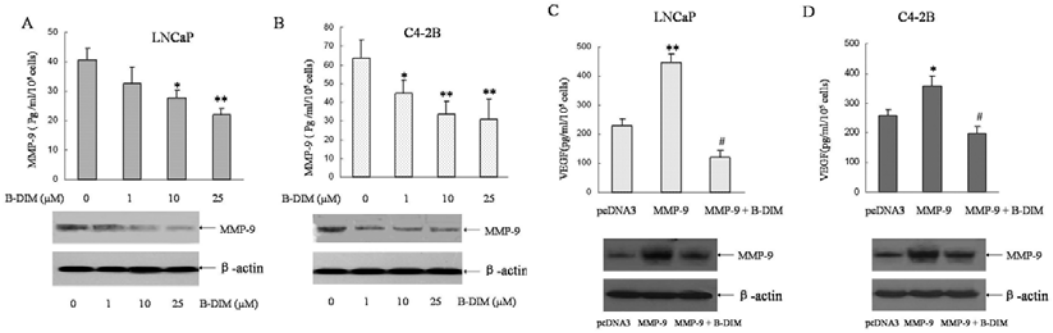


Figure 41. *B-DIM repressed secretion and expression of MMP-9 and regulation of VEGF release by MMP-9. (A) LNCaP and (B) C4-2B cells were cultured in serum free medium and treated with B-DIM. The culture media were assayed for MMP-9. The cell lysates were subjected to Western blotting using anti-MMP-9 antibody. (*, p < 0.05 and **, p < 0.01 compared to without B-DIM control). (C) LNCaP and (D) C4-2B cells were transfected with MMP-9 plasmid or pcDNA3 control plasmid and treated with B-DIM in serum free media. The culture media were collected for VEGF assay. Cell lysates were subjected to Western blotting using anti-MMP-9 antibody. (*, p < 0.05 and **, p < 0.01 compared to pcDNA control, #, p < 0.01 compared to MMP-9 group)*

in conditioned media from LNCaP and C4-2B cells transfected with MMP-9 plasmid and treated with B-DIM. The results showed that MMP-9 transfection significantly increases VEGF levels from conditioned media compared to transfection of control plasmid in LNCaP and C4-2B cells. Importantly, B-DIM treatment for LNCaP and C4-2B cells transfected with MMP-9 plasmid dramatically reduced the release of VEGF (Figure 41C and 41D). Western blot analysis showed that B-DIM repressed MMP-9 expression in LNCaP and C4-2B cells transfected with MMP-9 plasmid. These results suggest that B-DIM could reduce bioavailability of VEGF through repressing MMP-9 expression.

4). uPA regulated the bioavailability of the VEGF and B-DIM repressed uPA expression in LNCaP and C4-2B cells.

uPA is involved in the angiogenesis via the release or activation of many angiogenic growth factors such as VEGF. We found that down-regulation of uPA by siRNA significantly repressed the release of VEGF in LNCaP and C4-2B cells (Figure 42A), suggesting that B-DIM may decrease the bioavailability of VEGF via repressing the uPA expression. We also found that B-DIM treatment significantly decreased the uPA expression in LNCaP and C4-2B cells compared to control (Figure 42B). These results suggest that B-DIM may regulate the bioavailability of bound-VEGF by suppressing MMP-9 and uPA expression to inhibit angiogenesis.

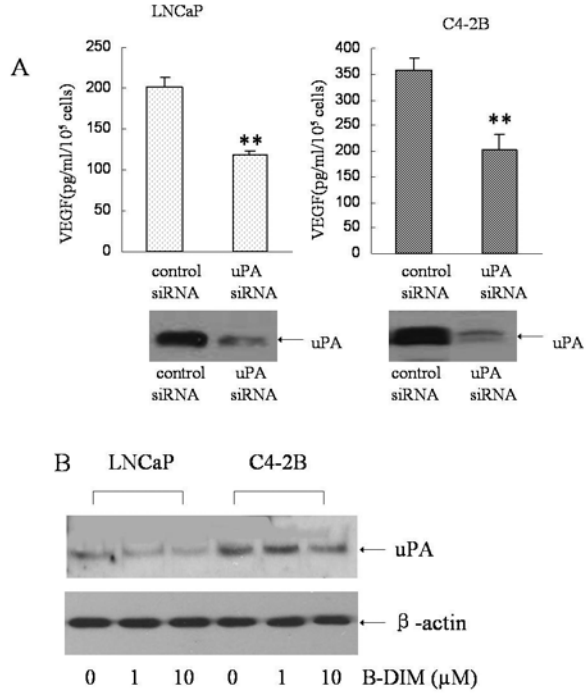


Figure 42. *uPA regulated the release of VEGF and B-DIM repressed uPA expression. (A) LNCaP and C4-2B cells were transfected with uPA siRNA. After 18h, the culture media were collected and assayed for VEGF. The cell lysates were subjected to Western blotting using anti-uPA antibody. (B) LNCaP and C4-2B cells were cultured in serum free medium for 24h, and then treated with B-DIM. Cell lysates from LNCaP and C4-2B cells were subjected to Western blotting using anti-uPA antibody.*

5). Reduced tube formation of HUVECs induced by conditioned media from LNCaP and C4-2B cells treated with B-DIM.

To determine whether conditioned media containing active VEGF from LNCaP and C4-2B cells could induce the tube formation of HUVECs, and whether conditioned media from LNCaP and C4-2B cells treated with B-DIM could reduce the tube formation, we performed the

tube formation assay in growth factor reduced Matrigel *in vitro*. Conditioned media (CM) from LNCaP and C4-2B cells (Figure 43A and 43B) as well as VEGF (Figure 43C) were able to significantly induce the tube formation of HUVECs in 6h incubation (compared to Figure 43C, medium only as negative control). To further explore whether VEGF in conditioned media may be the inducer of the tube formation of HUVECs, anti-VEGF neutralizing antibody was pre-incubated with conditioned media (CMAV) from LNCaP and C4-2B cells at 37°C for 1h then added into HUVECs. We found that anti-VEGF neutralizing antibody reduced the tube formation, suggesting that VEGF is the inducer of the tube formation induced by conditioned medium from LNCaP and C4-2B cells (Figure 43A). Importantly, we found that conditioned media from LNCaP and C4-2B cells treated with B-DIM inhibited tube formation (Figure 43A). These results suggest that B-DIM treatment inhibits tube formation by inhibiting secretion of pro-angiogenic factor VEGF in LNCaP and C4-2B cells.

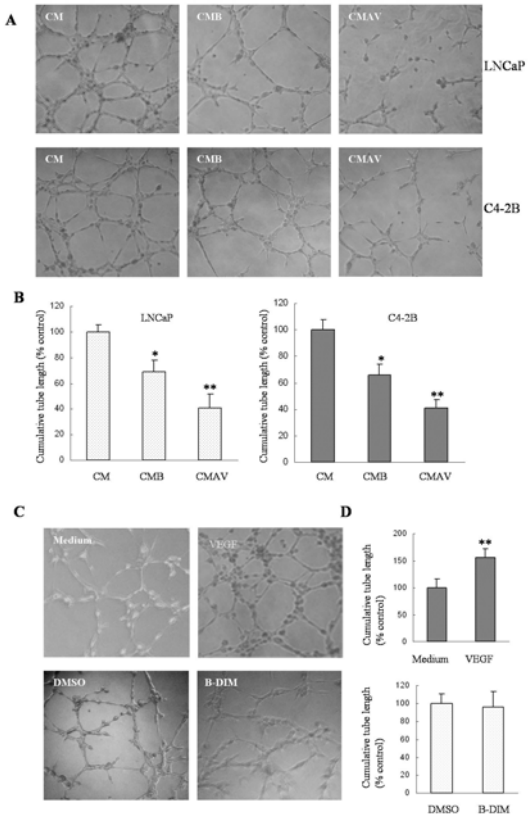


Figure 43. *Reduced tube formation of HUVECs induced by conditioned media from LNCaP and C4-2B cells treated with B-DIM. (A) Conditioned medium was from LNCaP and C4-2B cells treated with either DMSO (CM) or treated with 10 µmol/L B-DIM (CMB) in serum free medium for 24h and conditioned medium from LNCaP and C4-2B cells pre-incubated with 10 µg/ml VEGF neutralizing antibody at 37℃ for 1 hour (CMAV). The chamber was incubated for 6h. The well was photographed. (B) Image analysis of tubule/capillary length was carried out using Scion Image. (C) Medium only was used as negative control; 50 ng/ml VEGF was used as positive control. HUVECs were cultured in RPMI 1640 medium containing DMSO (DMSO) or 10 µmol/L of B-DIM (B-DIM). The chamber was incubated for 6h. The well was photographed. (D) Image analysis of tubule/capillary length was carried out using Scion Image. (**, $p < 0.01$).*

6). B-DIM inhibits in vivo angiogenesis.

We also investigated the effect of B-DIM on angiogenesis *in vivo* using a subcutaneously implanted Matrigel plug assay. Histological analysis showed that a larger number of endothelial cells were well organized into capillary or large vascular cavities engorged with red blood cells in the control group, whereas B-DIM treatment strongly inhibited the formation of large vessels (Figure 44A). In addition, B-DIM treatment also significantly reduced the vessel number (Figure 44B left panel), vascular area (middle panel) and total length of vascular perimeter (right panel) relative to the control group. These results provide strong evidence in support of the anti-

angiogenic activity of B-DIM *in vivo*, which is believed to be partially responsible for the anti-tumor activity of B-DIM.

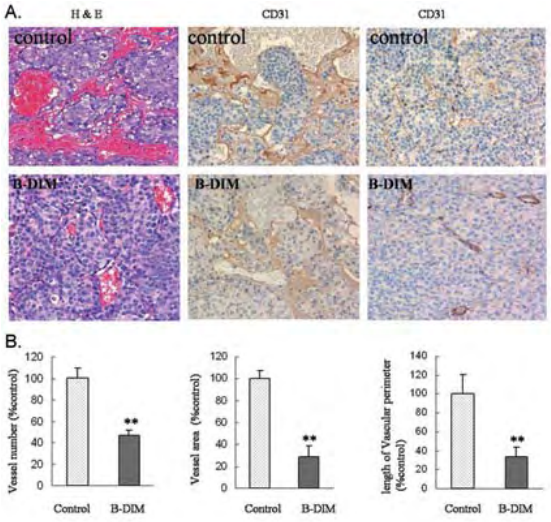


Figure 44. *B-DIM inhibited LNCaP and C4-2B cell in vivo angiogenesis. (A) C4-2B cells in Matrigel were injected subcutaneously into the bilateral flanks of each mouse. The control group of mice was gavaged with the vehicle (sesame oil), while the other group of mice was gavaged with B-DIM (5 mg/kg body weight, once a day) for 4 weeks. Matrigel plugs with tumor cells were harvested, fixed, and embedded. Matrigel plug slices were stained with H & E (left panel) and anti-CD31 antibody (middle and right panel). (B) Image analyses of the area of CD31-positive vascular, vascular number and length of vascular perimeter in each of the entire field were carried out using software image analysis program (**, p < 0.01 compared to control n = 4).*

7). B-DIM inhibited LNCaP and C4-2B cell invasion.

The levels of VEGF, MMP-9 and uPA are known to correlate with prostate cancer invasion and metastasis. We performed invasion assay. The results showed that 10 and 25 μM of B-DIM significantly inhibited LNCaP (Figure 45A) and C4-2B (Figure 45B) cell invasion. These results suggest that B-DIM inhibits LNCaP and C4-2B cell invasion by decreasing expression or bioavailability of VEGF via inhibiting matrix-degrading proteases such as uPA and MMP-9.

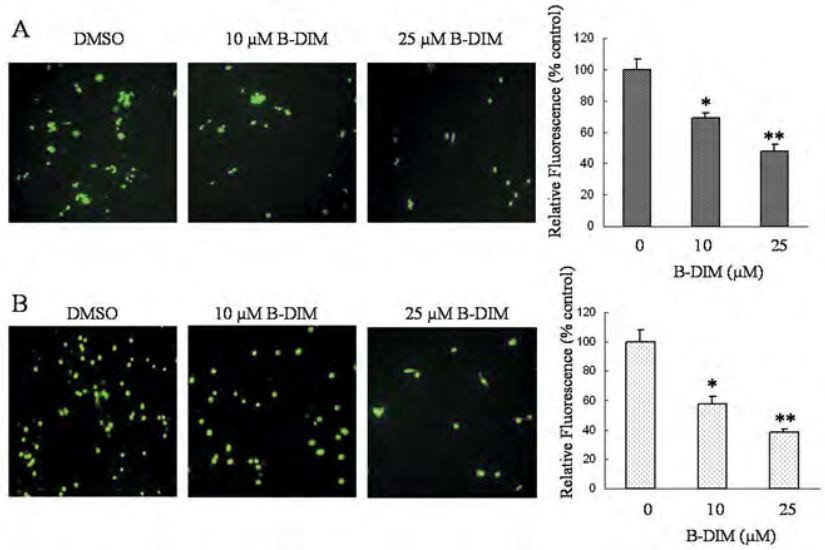


Figure 45. *B-DIM inhibited LNCaP and C4-2B cell invasion. (A) LNCaP and (B) C4-2B cells with serum free media containing 10 and 25 μM of B-DIM or DMSO were seeded into the upper chamber of the system. Bottom wells were filled with complete media. After 24h incubation, the cells invaded through the Matrigel membrane were stained with 4 μg/ml Calcein AM. The fluorescently labeled cells were photographed under a fluorescence microscope.*

These results suggest that B-DIM could down-regulate NF-κB downstream genes including VEGF, uPA, and MMP-9, leading to the inhibition of angiogenesis and invasion in prostate cancer. These data will be published in Cancer Research in April 1, 2007 [(20); please see Appendix 10]. Our published review articles and book chapter [(21, 22); please see

Appendices 11 and 12] are also attached to provide additional results that we have obtained from investigating effects of I3C/DIM on Akt and NF-κB pathways.

Conclusion: The task-3 has been fully completed.

Task-4. Task-4 is focused on to complete all data analysis, manuscript writing and for the development of novel ideas that may be submitted to Federal Agencies for further continued funding to test (a) whether I3C/DIM could be an effective agent for the prevention and/or for the treatment of tumors in animal models, (b) whether I3C/DIM pre-exposure of prostate cancer cells will sensitize these cells to commonly available chemotherapeutic agents, and finally (c) whether I3C/DIM could be an effective agent for the prevention and/or treatment of human prostate cancer.

We have analyzed all data demonstrated above and written manuscripts based on the results that we have obtained. Altogether, thirteen articles, book chapter, or meeting abstracts demonstrating the effects and molecular mechanisms of I3C/DIM have been published.

Conclusion: The task-4 has been fully completed.

Key Research Accomplishments

- Determined the different effects of I3C/DIM on the inhibition of cell growth and induction of apoptosis in PC-3, LNCaP, and C4-2B prostate cancer cells and non-tumorigenic CRL-2221 prostate epithelial cells.
- Measured the protein level of genes (Akt, pAkt, NF-κB, FOXO3a, AR, etc) related to the control of cell growth and apoptosis in prostate cancer cells.
- Gene expression profiling of I3C and DIM treated prostate cancer cells by microarray analysis. Cluster analysis of the expression of genes related to NF-κB inactivation, cell cycle arrest, apoptosis, transcript regulation, and other physiological processes in prostate cancer cells exposed to I3C and DIM.
- Determined the effect of I3C and DIM on NF-κB DNA binding activity in PC-3, LNCaP, and C4-2B prostate cancer cells and non-tumorigenic CRL-2221 prostate epithelial cells by EMSA.
- Determined the effects of I3C and DIM on Akt kinase activity in PC-3 and LNCaP prostate cancer cells and non-tumorigenic CRL-2221 prostate epithelial cells by immunoprecipitation, kinase assay, and Western Blot analysis.
- Demonstrated the cross-talk between Akt and NF-κB in PC-3, LNCaP, and C4-2B prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the effects of DIM and I3C on Akt and NF-κB pathways in Akt transfected PC-3, LNCaP, and C4-2B prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the effects of DIM and I3C on the induction of apoptosis in Akt transfected PC-3 and LNCaP prostate cancer cells by transfection, ELISA, and Western Blot analysis.
- Determined the inhibitory effect of B-DIM, a specially formulated DIM with greater bioavailability, on cell growth in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the effect of B-DIM on the induction of apoptosis in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the inhibitory effect of B-DIM on the expression of AR and PSA in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the inhibitory effect of B-DIM on the AR nuclear translocation in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the cross-talk between Akt, NF-κB, and AR in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells by transfection, luciferase assay, and EMSA.

- Determined the inhibitory effects of B-DIM on Akt, NF-κB, and AR pathways in Akt transfected LNCaP and C4-2B prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the down-regulation of Akt, NF-κB, and AR by B-DIM leading to the cell growth inhibition and apoptotic death in LNCaP and C4-2B cells.
- Determined the effects of I3C and DIM on the sensitization of prostate cancer cells to cell death induced by chemotherapeutic agents, cisplatin and Taxotere.
- Conducted IKKβ gene transfection study and investigated the effects and the molecular mechanisms of I3C and DIM action on the regulation of NF-κB pathway mediated by IKKβ.
- Conducted IKKα gene transfection study and investigated the effects and the molecular mechanisms of I3C and DIM action on the regulation of NF-κB pathway mediated by IKKα.
- Conducted MEKK and NIK gene transfection studies and investigated the effects of I3C and DIM on the regulation of NF-κB pathway mediated by MEKK and NIK.
- Determined the inhibitory effects of B-DIM on the phosphorylation of FOXO3a, the FOXO3a binding to AR promoter, and the expression of AR in LNCaP and C4-2B prostate cancer cells.
- Determined the effect of B-DIM on the up-regulation of FOXO3a binding to $p27^{KIP1}$ promoter and the expression of $p27^{KIP1}$.
- Determined that B-DIM down-regulated β-catenin, up-regulated the phosphorylation of β-catenin, and inhibited β-catenin nuclear translocation.
- Demonstrated the regulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling by B-DIM leading to cell growth inhibition and apoptotic death in LNCaP and C4-2B prostate cancer cells.
- Determined the inhibitory effects of B-DIM on the expression and secretion of NF-κB down-stream genes including VEGF, uPA, and MMP-9 in LNCaP and C4-2B prostate cancer cells.
- Determined the inhibitory effects of B-DIM on the bioavailability of VEGF mediated through the inhibition of uPA and MMP-9 in LNCaP and C4-2B cells.
- Demonstrated the down-regulation of VEGF, uPA, and MMP-9 by B-DIM leading to the inhibition of angiogenesis and invasion in LNCaP and C4-2B prostate cancer cells.

Reportable Outcomes

1. Kong D, Li Y, Wang Z, Banerjee S, Sarkar FH. Inhibition of angiogenesis and invasion by 3, 3'-diindolylmethane is mediated by NF-kappaB downstream target genes MMP-9 and uPA regulated bioavailability of VEGF in prostate cancer. Cancer Res 2007;(In press).

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4. Li Y, Wang Z, Kong D, Dou QP, Sheng S, Reddy GP, Sarkar FH. Regulation of FOXO3a/beta-catenin/GSK-3beta signaling by 3,3'-diindolylmethane (DIM) contributes to inhibition of cell proliferation and induction of apoptosis in prostate cancer cells. JBC 2007;(Submitted).

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Conclusions

In conclusion, we have found that I3C/DIM significantly inhibited the activation of Akt in PC-3 and LNCaP prostate cancer cells. Moreover, we found that I3C/DIM also significantly inhibited the activity of NF-κB. However, we did not observe such inhibitory effects of I3C/DIM on non-tumorigenic CRL-2221 prostate epithelial cells. Most importantly, we found that I3C/DIM could inhibit NF-κB activation and AR transactivation in prostate cancer cells transfected with activated Akt. These results demonstrate that there is a crosstalk between Akt, NF-κB, and AR signaling pathways and that I3C/DIM could interrupt this crosstalk, leading to the induction of apoptosis. From transfection experiments, we found that I3C/DIM inhibited the expression of IKKβ, the subsequent phosphorylation of IκBα, and the nuclear localization and activation of NF-κB. By microarray analysis, we found that I3C/DIM could regulate the expression of genes, which control cell cycle, apoptosis, Akt and MAPK signal transduction. We have also found that I3C/DIM induced cell growth inhibition and apoptotic death could be partly due to the regulation of Akt/FOXO3a/β-catenin/AR signaling. By inactivating NF-κB, B-DIM also down-regulated NF-κB downstream genes including VEGF, uPA, and MMP-9, leading to the inhibition of angiogenesis and invasion. More importantly, I3C and DIM could sensitize prostate cancer cells to chemotherapeutic agents through the inhibition of NF-κB and Akt pathways. These results suggest that I3C and DIM could be potent agents for the prevention and/or treatment of prostate cancers, especially hormone refractory prostate cancer in combination with common chemotherapeutic agents in future studies.

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Appendices

Publications, abstracts, and manuscripts during the funding:

1. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C- and DIM-treated PC-3 human prostate cancer cells determined by cDNA microarray analysis. J Nutr 2003;133**:**1011-9.

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inhibition of cell proliferation and induction of apoptosis in prostate cancer cells. JBC 2007;(Submitted).

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Final financial report will be separately sent out by our institute.

Nutrient-Gene Interactions

Gene Expression Profiles of I3C- and DIM-Treated PC3 Human Prostate Cancer Cells Determined by cDNA Microarray Analysis[1]

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ABSTRACT Studies from our laboratory and others have shown that indole-3-carbinol (I3C) and its in vivo dimeric product, 3,3′-diindolylmethane (DIM), inhibit the growth of PC3 prostate cancer cells and induce apoptosis by inhibiting nuclear factor (NF)-κB and Akt pathways. To obtain comprehensive gene expression profiles altered by I3C- and DIM-treated PC3 cells, we utilized cDNA microarray to interrogate the expression of 22,215 known genes using the Affymetrix Human Genome U133A Array. We found a total of 738 genes that showed a greater than twofold change after 24 h of DIM treatment. Among these genes, 677 genes were down-regulated and 61 were up-regulated. Similarly, 727 genes showed a greater than twofold change in expression, with down-regulation of 685 genes and up-regulation of 42 genes in I3C-treated cells. The altered expressions of genes were observed as early as 6 h and were more evident with longer treatment. Upon cluster analysis, we found that both I3C and DIM up-regulated the expression of genes that are related to the Phase I and Phase II enzymes, suggesting their increased capacity for detoxification of carcinogens or chemicals. We also found that I3C and DIM down-regulated the expression of genes that are critically involved in the regulation of cell growth, cell cycle, apoptosis, signal transduction, Pol II transcription factor and oncogenesis. Real-time reverse transcription-polymerase chain reaction analysis was conducted to confirm the cDNA microarray data, and the results were consistent. We conclude that I3C and DIM affected the expression of a large number of genes that are related to the control of carcinogenesis, cell survival and physiologic behaviors. This may help determine the molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells; in addition, this information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer. J. Nutr. 133: 1011–1019, 2003.

KEY WORDS: • *indole-3-carbinol* • *3,3′-diindolylmethane* • *gene expression* • *microarray* • *prostate cancer cells.*

Epidemiologic studies have shown that a high dietary intake of fruits and vegetables protects against carcinogenesis in many tissues (1,2). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family including broccoli, cabbage, brussels sprouts and cauliflower appears to be most effective at reducing the risk of cancers (3,4). Indole-3-carbinol (I3C),[3] a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family. There is growing evidence showing that I3C has the potential to prevent or even treat a number of common cancers, especially those that are hormone-related (5,6). It has been reported that a diet rich in cruciferous vegetables or supplements of I3C caused regression of tumors or decreased the rate of growth in patients with recurrent laryngeal papillomatosis (7,8). The in vivo and in vitro studies have also demonstrated that I3C possesses anticarcinogenic effects in experimental animals and inhibits the growth of human cancer cells (9,10). Because of this information, interest in I3C as a cancer chemopreventive agent has increased greatly in the last few years.

I3C is chemically unstable in acidic environments and is rapidly converted in the stomach to a variety of condensation products. Among them, 3,3′-diindolylmethane (DIM) is a major acid condensation product of I3C in vitro and in vivo. Because of the ready conversion of I3C to DIM and other products under a variety of biological conditions, the biological effects of I3C may be attributable to both I3C and DIM. Experimental studies have revealed that DIM exhibits inhibitory effects on cancer cells by inhibiting cell growth and inducing apoptosis that are similar to the effects observed with I3C (11,12). It has been reported that DIM exerts its chemoprotective effects in estrogen-responsive tissues, and DIM-induced G_1 arrest occurs with up-regulation of $p21^{WAF1/CIP1}$ in

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[3] Abbreviations used: ATF, activating transcription factor; CBFβ, core binding factor β; CYP, cytochrome P_{450}; DIM, 3,3′-diindolylmethane; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; I3C, indole-3-carbinol; MAPK, mitogen-activated protein kinase; MAP2K, MAP kinase kinase; MIG, mitogen-inducible gene; NF, nuclear factor; PI3K, phosphatidylinositol-3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; ST16, suppressor of tumorigenicity 16; TFDP, transcription factor Dp-1; TGF-β, transforming growth factor-β.

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breast cancer cells, suggesting its inhibitory effects on hormone-related cancers (11,13).

Prostate cancer is the most common nondermatological carcinoma in the United States with an estimated 189,000 new cases and 30,200 deaths in 2002 (14). Up to 30% of men undergoing radical prostatectomy will relapse, often as a result of micrometastatic disease present at the time of surgery (15). Thus, there is a tremendous need for the development of mechanism-based and targeted strategies for prevention and treatment of prostate cancer. cDNA microarray analysis allows us to examine the expression of tens of thousands of genes that can be monitored simultaneously and rapidly and, in turn, provides an opportunity to determine the effects of anticancer agents on prostate cancer cells (16). The gene expression profiles of various types of cancers were analyzed using cDNA microarray (17,18). The alterations of gene expression profiles by several anticancer agents have also been reported (19,20). This information is likely to contribute to devising preventive and/or therapeutic strategies more accurately, and will help to determine the molecular mechanism(s) of action of chemopreventive and/or therapeutic agents.

Our previous studies showed that I3C inhibits the growth of PC3 prostate cancer cells and induces apoptosis by inhibiting the nuclear factor (NF)-κB and Akt signaling pathways, suggesting that I3C may serve as a preventive and/or therapeutic agent against prostate cancer (21,22). However, little is known about the global gene expression profiles of prostate cancer cells after I3C and DIM treatment; the precise molecular mechanism(s) by which I3C and DIM exert their tumor suppressive effects on prostate cancer is also unclear. In this study, we utilized the high throughput gene chip, which contains 22,215 known genes, to identify changes in gene expression in PC3 prostate cancer cells exposed to I3C and DIM.

MATERIALS AND METHODS

Cell culture and growth inhibition. PC3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. I3C (Sigma, St. Louis, MO) or DIM (LKT, St. Paul, Minnesota) was dissolved in dimethyl sulfoxide (DMSO) to make a 100 or 50 mmol/L stock solution, respectively. For growth inhibition, PC3 cells were treated with 30, 60 and 100 μmol/L I3C or 15, 30, and 60 μmol/L DIM for 1–3 d. Control PC3 cells received 0.1% DMSO for the same time periods. The cells were then incubated with MTT (0.5 g/L, Sigma, St. Louis, MO) at 37°C for 4 h and with DMSO at room temperature for 1 h. The spectrophotometric absorbance of the samples was measured using the ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 495 nm. The experiment was repeated three times and *t* tests were performed to determine whether cell growth was inhibited by the treatments.

cDNA microarray analysis. PC3 cells were treated with 60 μmol/L I3C or 40 μmol/L DIM for 6, 24 and 48 h. DIM is the in vivo dimeric product of I3C. The doses of I3C and DIM chosen for microarray experiment were close to the 50% inhibitory concentration. However, the biological relevance of these doses in relation to prevention or therapy has not been fully evaluated, although they may have therapeutic application. The rationale for choosing these time points was to capture the expression profiles of early-response genes, genes that may be involved in the onset of growth inhibition and apoptotic processes and, finally, genes that may act as performers for the induction of apoptosis. Total RNA from each sample was isolated by Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit and RNase-free DNase Set (QIAGEN, Valencia, CA) according to the manufacture's protocols. cDNA for each sample was synthesized using a Superscript cDNA Synthesis Kit (Invitrogen) and a T7-$(dT)_{24}$ primer instead of the oligo(dT) provided in the kit. Then, the biotin-labeled cRNA was transcribed in vitro from cDNA using a BioArray HighYield RNA Transcript Labeling Kit (ENZO Biochem, New York, NY) and purified using the RNeasy Mini Kit. The purified cRNA was fragmented by incubation in fragmentation buffer (200 mmol/L Tris-acetate pH 8.1, 500 mmol/L potassium acetate, 150 mmol/L magnesium acetate) at 95°C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to the Human Genome U133A Array (Affymetrix, Santa Clara, CA), which contains 22,215 human gene cDNA probes, and hybridized to the probes in the array. After washing and staining, the arrays were scanned using a HP GeneArray Scanner (Hewlett-Packard, Palo Alto, CA). Two independent experiments were performed to verify the reproducibility of results.

Microarray data normalization and analysis. The gene expression levels of samples were normalized and analyzed using Microarray Suite, MicroDB and Data Mining Tool software (Affymetrix). The signal value of the experimental array was multiplied by a normalization factor to make its mean intensity equivalent to the mean intensity of the control array using Microarray Suite software according to manufacturer's protocol. The absolute call (present, marginal, absent) and average difference of 22,215 gene expressions in a sample, and the absolute call difference, fold change and average difference of gene expressions between two or several samples were identified using the above-mentioned software. Statistical analysis of the difference in the mean expression of genes that indicated a greater than twofold change was performed repeatedly between treated and untreated samples using *t* tests. Average-linkage hierarchical clustering of the data was applied using the Cluster (23) and the results were displayed with TreeView (23). The genes showing altered expression were also categorized on the basis of their location, cellular component and reported or suggested biochemical, biological and molecular functions using Onto-Express (24). Genes that were not annotated or not easily classified were excluded from the functional clustering analysis.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. The total RNA prepared for microarray analysis was also used for RT-PCR analysis of selected genes. Total RNA (2 μg) from each sample was subjected to reverse transcription using a Superscript first strand cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol. Real-time PCR reactions were then

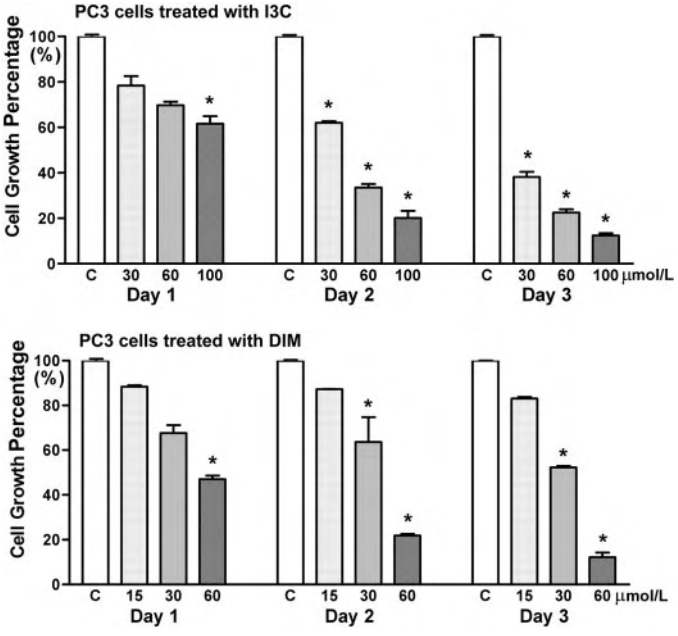

FIGURE 1 Effects of various doses of indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM) on the growth of PC3 cells for 3 d. Data are means ± SEM, $n = 3$. *Different from solvent control (C), $P < 0.05$.

carried out in a 25 μL reaction mixture (2 μL of cDNA, 12.5 μL of 2X SYBR Green PCR Master Mix, 1.5 μL of 5 μmol/L specific gene primer pair and 9 μL of H_2O) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The sequences of primers used in the real-time PCR reaction were described previously (25). The PCR program was initiated by 2 min at 50°C and 10 min at 95°C before 40 thermal cycles, each of 15 s at 95°C and 1 min at 60°C. Data were analyzed according to the comparative cycle threshold (Ct) method and were normalized by actin expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

RESULTS

Cell growth inhibition. The MTT assay showed that the treatment of PC3 prostate cancer cells with I3C or DIM resulted in dose- and time-dependent inhibitions of cell proliferation (**Fig. 1**), demonstrating the growth inhibitory effect of these compounds on PC3 cells. These results are consistent with our previously published results (22).

Regulation of mRNA expression. The gene expression profiles of PC3 cells treated with I3C or DIM were assessed

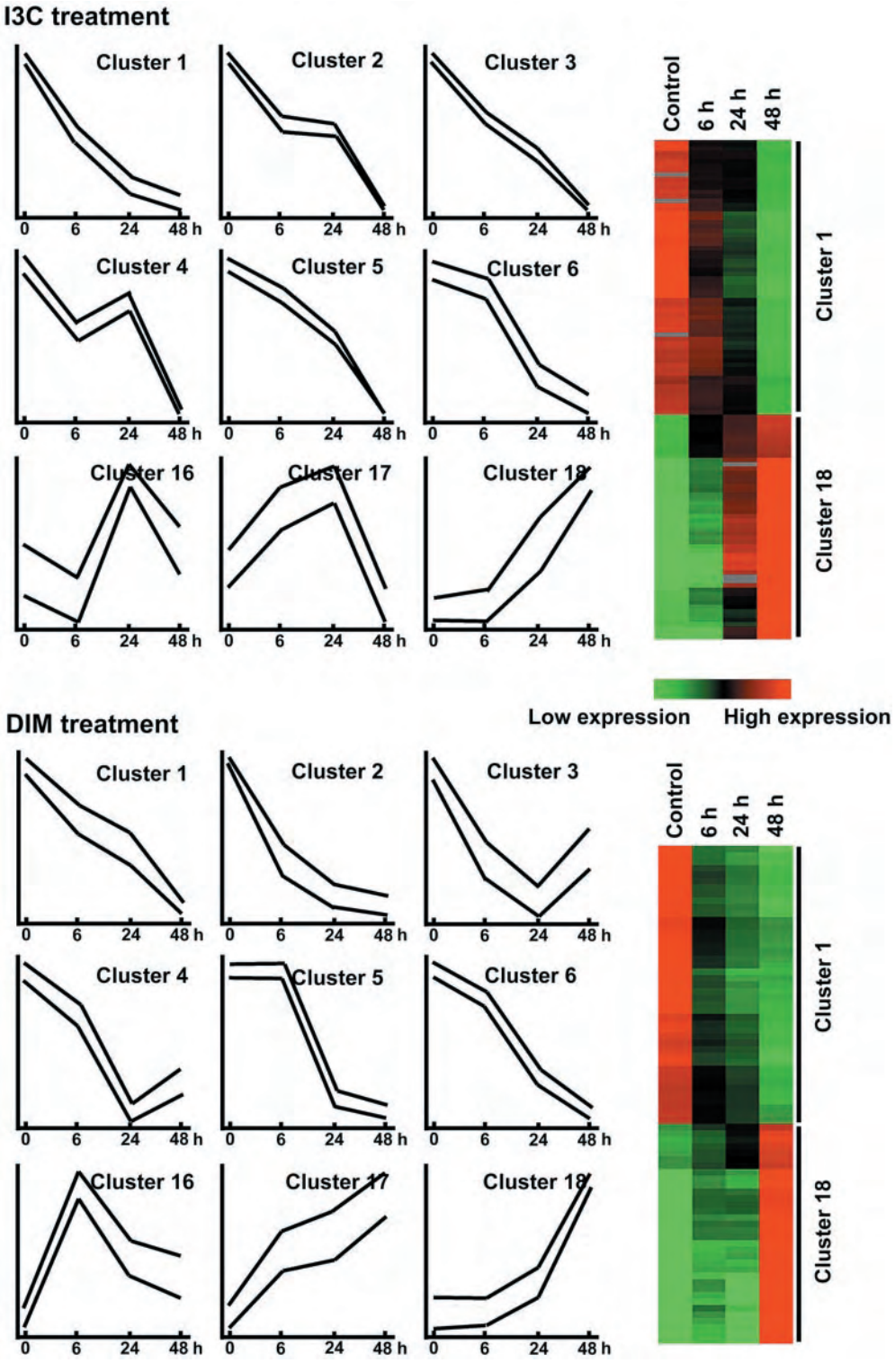

FIGURE 2 Cluster analysis of genes showing alterations in mRNA expression after indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM) treatment of PC3 cells. Cluster 1 and cluster 18 included the genes showing typical gradual decreases and increases in expression, respectively.

using cDNA microarray. Two independent experiments showed a total of 738 genes showing a greater than twofold change after 24 h of DIM treatment. Among these, 677 genes were down-regulated and 61 were up-regulated. Similarly, 727 genes showed a greater than twofold change in expression with down-regulation of 685 genes and up-regulation of 42 genes in I3C-treated cells. Clustering analysis showed 18 different types of expression alterations in DIM- or I3C-treated PC3 cells, respectively. Cluster 1 and cluster 18 included the genes showing typical gradual decreases and increases in expression, respectively. The altered expressions of genes occurred after only 6 h of I3C and DIM treatment, and were more evident with longer treatment (**Fig. 2**).

The altered genes were also subjected to cluster analysis according to location and cellular components. The genes showing altered expression were located primarily on chromosomes 1, 2, 3, 6, 7 and 12, and were responsible mainly for the transcription and translation of components of the nucleus and integral plasma membrane proteins. After clustering based on biological function, we found that I3C and DIM down-regulated mainly genes that are involved in the processes of signal transduction, oncogenesis, cell proliferation, antiapoptosis and transcription regulation from Pol II promoter. On the other hand, I3C and DIM up-regulated mainly genes that are related to Phase I and II biotransformation, signal transduction, induction of apoptosis, inflammation response and prostaglandin metabolism (**Tables 1**, **2** and **3**). When genes were classified by molecular function, I3C and DIM treatment

TABLE 1

Classification by function of genes with altered expression in PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) for 48 h[1]

Down-regulation	Genes, *n*	Up-regulation	Genes, *n*
Biological processes			
Signal transduction	56	Cell cycle checkpoint	3
Transcription regulation from Pol II promoter	18	Developmental processes	3
Cell adhesion	16	Protein phosphorylation	2
Cell proliferation	16	Small molecule transport	2
Oncogenesis	12	Central nervous system development	2
Cell cycle control	11	Signal transduction	2
Protein phosphorylation	10	Nonselective vesicle transport	1
Cell-cell signaling	8	Skeletal development	1
Protein modification	8	Immune response	1
RNA processing	8	Cell surface receptor linked signal transduction	1
Transcription regulation	6	Cell growth and maintenance	1
DNA replication	5	Stress response	1
Positive control of cell proliferation	5	Sodium transport	1
Receptor tyrosine phosphatase signaling	5	Cytostolic calcium ion concentration elevation	1
Transcription from Pol II promoter	5	G-protein linked receptor protein signaling	1
Drug resistance	4	Repression of transcription from Pol II promoter	1
EGF receptor signaling pathway	4	Induction of apoptosis	1
Translational regulation, initiation	4	Excretion	1
JNK cascade	4	Apoptosis	1
Cell motility	3	Spermatid development	1
JAK-STAT cascade	2	Male meiosis	1
Antiapoptosis	2	Peripheral nervous system development	1
Activation of JUN kinase	2	Antimicrobial humoral response	1
TGFβ receptor signaling pathway	2	Cysteine metabolism	1
MAP3K cascade	1	Brain development	1
Molecular functions			
Transcription factor	13	Calcium binding	2
DNA binding	12	Serpin	2
Protein serine/Threonine kinase	12	Cytochrome P_{450}	1
Adenosinetriphosphatase	9	Tumor suppressor	1
Transcription co-activator	9	Serine C-palmitoyltransferase	1
Protein kinase	9	Receptor signaling protein	1
RNA binding	8	Interleukin receptor	1
RNA polymerase II transcription factor	6	Diamine *N*-acetyltransferase	1
Transcription co-repressor	5	Receptor	1
Translation factor	5	Ligand	1
Signal transduction	3	G-protein linked receptor	1
Receptor protein tyrosine phosphatase	3	Protein binding	1
Transcription activating factor	3	Transcription co-repressor	1
Apoptosis inhibitor	2	Tryptophan transporter	1
JAK pathway signal transduction adaptor	2	Purine nucleotide transporter	1
Epidermal growth factor receptor	2	ATP-binding cassette (ABC) transporter	1
MAP3K	2	ATP binding	1
Inositol-1,4,5-triphosphate receptor	2	Transporter	1
Type II TGFβ receptor	2	Enzyme inhibitor	1
Cytokine	2	Heat shock protein	1
Cell cycle regulator	1	Chaperone	1
Transcription co-factor	1	Ligand-dependent nuclear receptor	1

[1] Abbreviations used: EGF, epidermal growth factor; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; TGF, transforming growth factor.

TABLE 2

Classification by function of genes with altered expression in PC3 cells treated with 40 μmol/L 3,3′-diindolylmethane (DIM) for 48 h[1]

Down-regulation	Genes, *n*	Up-regulation	Genes, *n*
Biological processes			
Signal transduction	42	Inflammatory response	4
Oncogenesis	16	Signal transduction	4
Cell proliferation	16	Prostaglandin metabolism	2
Cell-cell signaling	15	Cell cycle checkpoint	2
Cell cycle control	13	G-protein linked receptor protein signaling	2
Transcription regulation from Pol II promoter	11	Protein complex assembly	1
Transcription from Pol II promoter	11	Immune response	1
RNA processing	9	Potassium transport	1
Protein phosphorylation	9	Defense response	1
Cell adhesion	9	Physiological processes	1
Cell motility	9	Response to pathogenic fungi	1
Positive control of cell proliferation	6	Proteolysis and peptidolysis	1
DNA replication	5	Complement activation	1
RNA splicing	5	Glucose metabolism	1
Protein complex assembly	5	Developmental processes	1
Pathogenesis	5	Stress response	1
Cytostolic calcium ion concentration	5	Homophilic cell adhesion	1
Antiapoptosis	5	Small molecule transport	1
Immune response	5	Heavy metal response	1
Transcription from Pol I promoter	3	Proton transport	1
JNK cascade	2	Virulence	1
Transcription	2	Receptor protein tyrosine kinase signaling	1
Transcription from Pol III promoter	2	Phosphatidylinositol bisphosphate hydrolysis	1
EGF receptor signaling pathway	1	Cytostolic calcium ion concentration elevation	1
Transcription initiation from Pol II promoter	1	Circulation	1
Activation of MAPK	1	Cell surface receptor linked signal transduction	1
Molecular functions			
RNA binding	20	Cytochrome P_{450}	3
Transcription factor	16	Electron transporter	3
DNA binding	15	Protein binding	3
Transcription co-activator	10	Calcium binding	2
RNA polymerase II transcription factor	9	Cystine/glutamate antiporter	1
Protein binding	8	Chaperone	1
Signal transduction	8	Interleukin-1 receptor	1
Protein serine/Threonine kinase	8	Calcium-activated potassium channel	1
Adenosinetriphosphatase	7	Prostaglandin-endoperoxide synthase	1
Cytokine	7	GTP binding	1
Transcription activating factor	6	Serine-type peptidase	1
Heat shock protein	6	Complement factor D	1
Cell cycle regulator	3	Double-stranded RNA binding	1
Specific RNA pol II transcription factor	3	Adenosine deaminase	1
Apoptosis inhibitor	3	RNA binding	1
Transforming growth factorβ receptor	3	Aldo-keto reductase	1
General RNA pol II transcription factor	2	Phosphoenolpyruvate carboxykinase	1
G_1/S-specific cyclin	2	Tumor suppressor	1
Tumor antigen	2	Adenosinetriphosphatase	1
Receptor protein tyrosine phosphatase	2	General RNA polymerase II transcription factor	1
Cyclin	2	Bradykinin receptor	1
Growth factor	1	Chemokine receptor	1
Interleukin-8 receptor ligand	1	Cytokine	1
MAP kinase kinase	1	Enzyme	1

[1] See Table 1 for abbreviations.

down-regulated those responsible for RNA binding, DNA binding, transcription factors, transcription coactivators and protein kinases including mitogen-activated protein kinase (MAPK), MAP2kinase, MAP3kinase, c-Jun N-terminal protein kinase and up-regulated tumor suppressors, transcription corepressors and cytochrome P_{450} enzymes (Tables 1–3). However, I3C and DIM also showed some differential effects on gene expression profiles (Table 3).

Target verification by RT-PCR. To verify the alterations of gene expression at the mRNA level, which appeared on the microarray, we chose six genes [transforming growth factor-β (TGF-β)2, $p57^{KIP2}$, cytochrome b5, eukaryotic protein synthesis initiation factor 4, thrombospondin 1 and neuropilin 1] with varying expression profiles for real-time RT-PCR analysis. The results of real-time RT-PCR analysis for these selected genes were consistent with the microarray data (**Figs. 3** and **4**). Gene expression alterations were similar by real-time RT-PCR analysis, although the fold changes in the expression level differed somewhat in the two analytical methods. These results support the findings obtained from microarray experiments, and also suggest that I3C and DIM regulate the transcription of genes that are

TABLE 3

Fold changes of specific genes in PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) or 40 μmol/L 3,3′-diindolylmethane (DIM) for 6, 24 or 48 h[1,2]

	I3C			DIM		
Gene name	6 h	24 h	48 h	6 h	24 h	48 h
Phase I and II enzymes						
NM_000499.2 Cytochrome P_{450} (CYP1A1)	1.4	3.7	5.2	1.8	2.2	5.4
M25813.1 Human P_{450}c21 gene	NC[3]	NC	NC	NC	NC	3.5
N21019 Cytochrome $P4_{50}$	NC	NC	NC	NC	5.2	3.5
AU154504 Cytochrome P_{450}	NC	NC	NC	NC	2.3	3.4
AU144855 Cytochrome P_{450}	NC	NC	NC	NC	2.3	3
NM_000104.2 Cytochrome P_{450} (CYP1B1)	NC	NC	NC	2	1.9	2.2
AB018580.1 Aldo-keto reductase	NC	NC	3.6	NC	2.9	9.2
NM_005589 Methylmalonate-semialdehyde dehydrogenase	NC	NC	NC	NC	NC	2.4
AK000550.1 Phospholipase A_2 β	NC	NC	NC	NC	5	NC
NM_000853.1 Glutathione *S*-transferase theta 1	NC	NC	2.1	NC	NC	2.1
NM_019886.1 Carbohydrate sulfotransferase 7	NC	NC	NC	1.7	1.4	2
NM_012200.2 Glucuronosyltransferase I	NC	NC	NC	12.6	NC	NC
AB009598 Glucuronyltransferase I	NC	NC	NC	2.3	NC	NC
Cell growth, cell cycle, apoptosis						
K03193.1 Human epidermal growth factor receptor	−1.4	−2.6	−4.3	1	−2.7	−4.1
BF061658 Transforming growth factor	NC	−1.8	−4	−1.2	−5.1	−6.4
NM_003238.1 Transforming growth factor	NC	−2	−6.9	−1.2	−3.5	−4.9
NM_000358.1 Transforming growth factor	−1	−1.1	−2.1	−1	−3.3	−3.7
M19154.1 Human transforming growth factor-β-2	NC	−2	−5.4	−1	−3	−4.7
NM_002006.1 Fibroblast growth factor 2	NC	−1.9	−4.9	NC	−1.8	−1.3
AI434345 Activating transcription factor 1	−1.2	−1.4	−3.8	NC	−1.8	−2.2
Z24725.1 Mitogen-inducible gene mig-2	NC	−1.4	−2.2	−1.1	−2.4	−2
NM_004702.1 Cyclin E2 (CCNE2)	NC	NC	−1.7	NC	−2.3	−4.3
AF112857.1 Cyclin E2 splice variant 1	NC	NC	−1.3	−1	−2.8	−5.4
NM_021960.1 Myeloid cell leukemia sequence (BCL2-related)	−1.1	NC	−1.5	−1	−2.3	−3.4
NM_004049.1 BCL2-related protein A1 (BCL2A1)	NC	NC	−1.4	−1.2	−2.1	−3.5
D64137 KIP2 Gene for Cdk-inhibitor p57KIP2	NC	2.2	4.3	2.3	4.5	4.5
NM_000076.1 Cyclin-dependent kinase inhibitor 1C(p57Kip2)	NC	1.6	1.1	2.1	3.4	4.7
NM_030579.1 Cytochrome b5	−1.4	−1.4	−6.9	NC	−1.6	−1.9
Cell signal transduction pathways						
NM_003954.1 MAP kinase kinase kinase 14	−1.1	−1.6	−2.3	−1.1	−2.7	−3.9
NM_002756.1 MAP kinase kinase 3 (MAP2K3)	−1.2	1.1	−1.7	NC	−2.3	−2.7
NM_002376.1 MAP regulating kinase 3 (MARK3)	−1.3	−1.7	−3.3	NC	−2.2	−3.1
NM_003010.1 MAP kinase kinase 4 (MAP2K4)	−1.3	−1.7	−4.1	−1.1	−2	−2.9
NM_003618.1 MAP kinase kinase kinase kinase 3 (MAP4K3)	−1.4	−2	−6.8	NC	−2	−2.4
NM_006218.1 Phosphoinositide-3-kinase	−1.4	−1.2	−2.1	NC	NC	NC
NM_002646.1 Phosphoinositide-3-kinase	−3	−1.4	−2.4	NC	NC	NC
Transcription factors, oncogenesis, angiogenesis, other						
NM_007111.1 Transcription factor Dp-1 (TFDP1)	−1.2	−1.3	−3.8	NC	−2.1	−3.7
U62296.1 Transcription factor NF-YC	−1.2	−1.5	−3.4	NC	−2	−2.1
BE966878 Eukaryotic protein synthesis initiation factor 4	NC	NC	NC	−1.1	−1.8	−2.1
NM_001755.1 Core-binding factor, β subunit (CBFB)	−1.2	−1.6	−2.9	NC	−2	−2.1
NM_006850.1 Suppression of tumorigenicity 16 (ST16)	2.2	3.6	2.4	1.2	1.7	2.4
AI812030 Thrombospondin 1	NC	−1.9	−4.7	−1	−3	−4.3
BE999967 Thrombospondin 1	−1	−1.4	−3.9	−1.2	−3.3	−5
BE620457 Neuropilin 1	−1.6	−1.9	−8.2	NC	NC	NC
M92934.1 Connective tissue growth factor	NC	NC	NC	−1.9	−14.7	−19.8

[1] Positive values represent increases, negative values are decreases.
[2] Abbreviations: Cdk, cyclin-dependent kinase; MAP, mitogen-activated protein; NF, nuclear factor.
[3] No change.

involved in the physiologic processes of prostate cancer cells.

DISCUSSION

Our results from cDNA microarray provided a genome-wide analysis of the cellular response to I3C and DIM treatments. Cellular responses to any antiproliferative agents involve modulation of complex pathways that ultimately determine whether a cell survives or dies. Our data revealed that the alterations of biological processes and molecular functions in I3C- and DIM-treated PC3 cells are complex and are likely to be mediated by a variety of regulatory pathways. First, we found that I3C and DIM regulated the expression of phase I and II enzymes in PC3 prostate cancer cells, suggesting the anticarcinogenic effects of I3C and DIM. Carcinogenesis is a multistage process, and there is great opportunity for intervention to stop, revert or delay the carcinogenic process. One

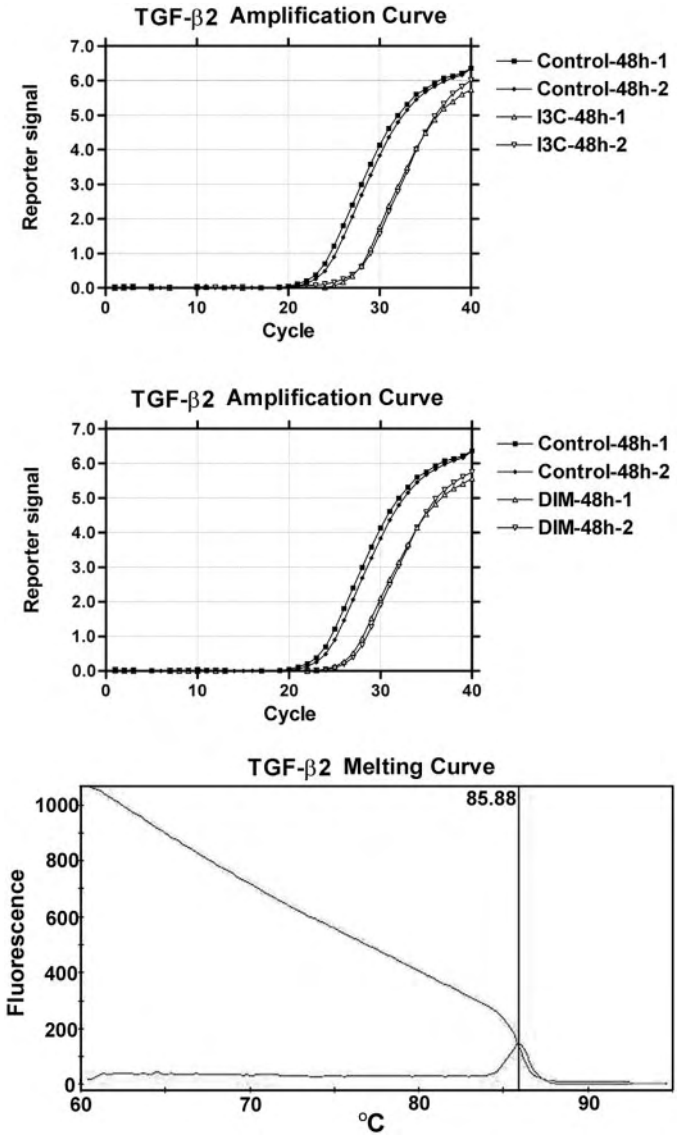

FIGURE 3 Real-time reverse transcription-polymerase chain reaction (RT-PCR) amplification curves showing the amplification of transforming growth factor (TGF)-β2 from RNA of PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) (*upper panel*) or 40 μmol/L 3,3′-diindolylmethane (DIM) (*middle panel*) for 48h. 48h-1 and 48h-2 are duplicated experiments for 48h treatment sample. *Lower panel*: the real-time RT-PCR melting curve showing the product is pure (only one peak).

anticarcinogenic action is the modulation of metabolism of carcinogens, including inhibition of procarcinogen activation, induction of detoxification and blocking of reactive metabolites (3). The molecules involved in this modulation are phase I and phase II biotransformation enzymes. The most important phase I enzymes are the cytochrome P_{450} (CYP) enzymes. These enzymes oxidize carcinogens and make them more hydrophilic as well as susceptible to detoxification (3,26). Phase II metabolism comprises detoxification and conjugation reactions, making phase I metabolites more polar and readily excretable (3,26). We found that I3C and DIM up-regulated the expression of the phase I enzyme CYP1A1 and the phase II enzymes, glutathione *S*-transferase theta 1 and aldo-keto reductase, in PC3 prostate cancer cells, suggesting their increased capacity for detoxification and inhibition of carcinogens. It has been reported that both I3C and DIM elevated the activity of phase I and phase II enzymes in rat liver (3). Therefore, our microarray data are consistent with the in vivo experimental reports published by other investigators and provide molecular evidence that I3C and DIM may serve as chemopreventive agents against prostate cancer because of their ability to induce phase I and phase II enzymes.

Because DIM is one of the major in vivo dimeric products of I3C, we also found that the molecular response to both I3C and DIM in PC3 prostate cancer cells involved mainly inhibition of genes that are related to cell growth, cell cycle control, apoptosis, signal transduction, oncogenesis, transcription regulation and protein phosphorylation in addition to the induction of phase I and II enzymes. Epidermal growth factor receptor (EGFR) is a cell membrane protein that is overexpressed in many cancers that have a poor prognosis. This cell membrane protein has been considered to be an excellent target for antitumor therapy (27). TGF-β and fibroblast growth factor (FGF) are multifunctional and essential to the survival of cancer cells. They play important roles in promoting cell growth and angiogenesis (28,29). We observed down-regulation of the EGFR, TGFβ2 and FGF by I3C and DIM treatment, demonstrating the inhibitory effects of I3C and DIM on the growth of PC3 prostate cancer cells. Cyclin E, activating transcription factor (ATF) and mitogen-inducible gene (MIG) regulate cell cycle progression, and BCL2 inhibits apoptotic cell death (30–33). The overexpression of ATF has also been observed in metastatic cancer cells (34). $p57^{KIP2}$ is a tumor suppressor that inhibits cyclin-dependent kinase and results in cell cycle arrest (35). Our results showed that I3C and DIM inhibited the expression of cyclin E2, ATF5, MIG-2 and BCL2, and induced the expression of $p57^{KIP2}$, which may lead to the induction of cell cycle arrest and apoptosis. Taken together, I3C and DIM appear to modulate the expression of several genes that may contribute to the observed cell growth inhibition as demonstrated by the MTT cell growth inhibition assay.

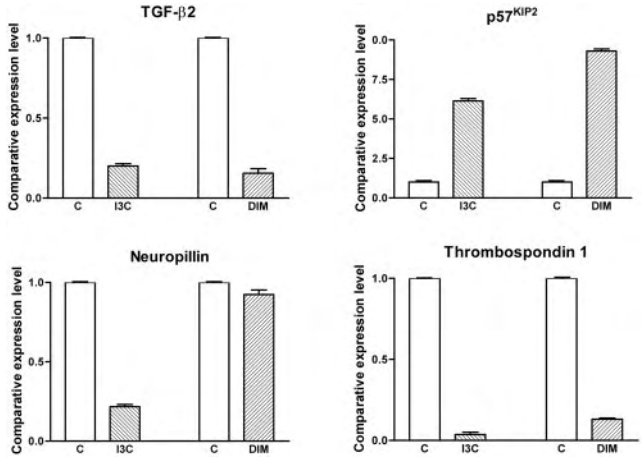

FIGURE 4 Real-time reverse transcription-polymerase chain reaction analysis of selected genes showing alterations in mRNA expression of the specific genes in PC3 cells treated with 60 μmol/L indole-3-carbinol (I3C) or 40 μmol/L 3,3′-diindolylmethane (DIM) for 48 h compared with control PC3 cells. C represents the solvent control; TGF, transforming growth factor. Data are means $\pm$ SEM, $n = 3$; calculated by the comparative cycle threshold method.

Cell signal transduction pathways are important for cell survival. Recently, the MAPK and phosphatidylinositol-3-kinase (PI3K)/Akt pathways have received much attention in cancer research and are believed to be excellent targets for cancer prevention and therapy. The MAPK pathway consists of a three-tiered kinase core in which a MAP3K activates a MAP2K that activates a MAPK, resulting in the activation of NF-κB, cell growth and cell survival (36,37). We observed down-regulation in the expression of MAP2K3, MAP2K4, MAP4K3, and MARK3 by I3C and DIM treatments, suggesting that I3C and DIM have inhibitory effects on the MAPK pathway, resulting in the abrogation of cancer cell survival. PI3K/Akt pathway is another important signal transduction pathway and plays a critical role in controlling cell survival and apoptosis (38). From the gene expression profiles of PC3 cells exposed to I3C, we found down-regulation of PI3K expression, suggesting that I3C could induce apoptosis and inhibit cancer cell survival by altering the PI3K/Akt pathway. These findings from cDNA microarray gene analysis are consistent with our previous report showing that I3C inhibits cancer cell growth and induces apoptosis by inhibiting the NF-κB and Akt signal pathways (21,22).

Several Pol II transcription factors, including transcription factor Dp-1 (TFDP) and NF-YC, play important roles in cell transcription (39,40). TFDP1 overexpression leads to up-regulation of cyclin E, which encodes a positive regulator for cell cycle G_1/S transition (39). The overexpression of these transcription factors has also been related to oncogenesis. In addition, core binding factor β (CBFβ) and suppressor of tumorigenicity 16 (ST16) are also involved in oncogenesis. CBFβ forms a fusion protein with other gene products and promotes oncogenesis (41), whereas ST16 suppresses oncogenesis (42). Our results showed that I3C and DIM down-regulated the expression of TFDP1, NF-YC, DKC1, cyclin E and CBFB, and up-regulated ST16 expression, suggesting that I3C and DIM can inhibit transcription and oncogenesis, and could also induce G_1 arrest, as demonstrated by our previous study (22).

It is important to note, however, that I3C and DIM also had some differential effects on the gene expression profile, which is perhaps expected. We observed that DIM but not I3C induced the expression of other phase II enzymes including methylmalonate-semialdehyde dehydrogenase, phospholipase A2, carbohydrate sulfotransferase 7 and glucuronosyltransferase I, suggesting that DIM may have a more inhibitory effect on oncogenesis than I3C.

In summary, the goal of the present study was to analyze the gene expression profiles of PC3 prostate cancer cells after exposing them to I3C and DIM. Both I3C and DIM changed the expression of a large number of genes that are related to the control of carcinogenesis, cell survival and physiologic behaviors. This may help determine the molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells; such information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer.

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Significance of indole-3-carbinol and its metabolite in human cancers

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Abstract: Epidemiological studies have demonstrated that a significant difference exists in the incidence of cancer among different ethnic groups. This difference is known to be partly attributed to dietary factors. Indole-3-carbinol (I3C) and its in vivo dimeric product 3,3′-diindolylmethane (DIM) are produced from naturally occurring glucosinolates in the family Cruciferae. They have received much attention as dietary components that have an inhibitory affect on cancer. I3C and DIM up-regulate the expression of phase I and phase II enzymes, suggesting an increased role in the detoxification and inhibition of carcinogens. They also inhibit the growth of cancer cells through the modulation of genes that are related to the control of cell proliferation, cell cycle, apoptosis, signal transduction, oncogenesis, transcription regulation and protein phosphorylation. Moreover, I3C and DIM inactivate NF-κB, Akt and MAPK signalling pathways, all of which are believed to be potential targets in cancer prevention and therapy. Collectively, several studies have provided evidence for pre-clinical and clinical activities of I3C and DIM against some cancers. Hence, significant advances have been made to date that show I3C and DIM as promising agents for cancer chemoprevention and/or treatment. This review summarises the well accepted inhibitory effects of I3C and DIM on cancer cells and provides a comprehensive view on their molecular mechanism(s) of cancer chemoprevention.

Keywords: I3C, DIM, cancer, prevention, treatment

Introduction

Epidemiological studies have demonstrated that a significant difference exists in the incidence of cancer among different ethnic groups. This difference is known to be partly attributed to dietary factors. Studies from in vitro and in vivo experiments have shown that dietary factors regulate the processes of carcinogenesis, including the initiation, promotion and progression of human cancers (Meyskens 1992; Yang et al 2001). It has been reported that certain components of plant foods, such as fiber, isoflavone and hydrolysis products of glucosinolates, may be protective against cancers (Adlercreutz et al 1995; Steinmetz and Potter 1996; Verhoeven et al 1997). Indole-3-carbinol (I3C) is produced from naturally occurring glucosinolates, contained in a wide variety of plants including members of the family Cruciferae and, in particular, members of the genus *Brassica* (Broadbent TA and Broadbent HS 1998). I3C is biologically active, and is easily converted in vivo to its dimeric product 3,3′-diindolylmethane (DIM), which is also biologically active. Over one hundred glucosinolates have been identified, predominantly in vegetables of the family Cruciferae (Verhoeven et al 1997; Johnson 2002). In this family, 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 sulphonated oxime moiety, but differ in side chain R (Figure 1). Glucosinolate hydrolysis products make a significant contribution to the health benefit of brassicaceous vegetables (Tawfiq et al 1995; Johnson 2002). The enzyme myrosinase, which is found in plant cells and also in certain intestinal microflora, catalyses the hydrolysis of glucosinolates (Verkerk et al 1997; Johnson 2002). The glucosinolate hydrolysis products include equimolar amounts of aglucon, glucose and sulphate. The aglucones are unstable and undergo further reactions. The nature of aglucones depends primarily on the side chain of the glucosinolate (Tawfiq et al 1995; Verhoeven et al 1997). Glucosinolates with an indole side chain form indoles. The

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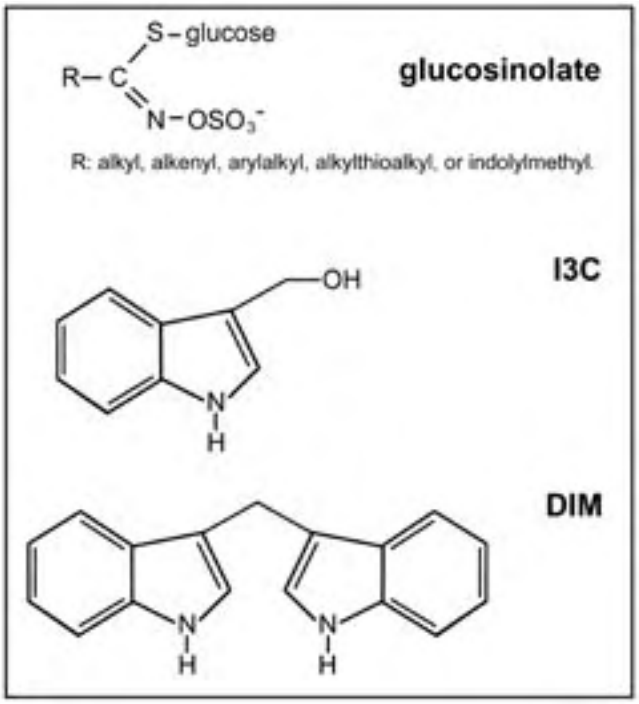

Figure 1 Molecular structure of glucosinolate.

most prevalent glucosinolate with an indole side chain is glucobrassicin, which is predominant in brassicaceous vegetables. When hydrolysis occurs, glucobrassicin forms an unstable isothiocyanate, which degrades to I3C (Verhoeven et al 1997; Broadbent TA and Broadbent HS 1998). I3C is the immediate precursor of DIM. Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form several derivatives (Dashwood et al 1994; Verhoeven et al 1997). DIM is the major derivative and condensation product of I3C (Figure 1), and its formation from I3C has been believed to be a likely prerequisite for I3C-induced anticarcinogenesis (Dashwood et al 1994).

It has been reported that I3C and DIM possess anticarcinogenic effects in experimental animals and inhibit the growth of human cancer cells (He et al 1997; Oganesian et al 1997; Cover et al 1998; Katdare et al 1998; Jin et al 1999; Chen DZ et al 2001; Murillo and Mehta 2001; Hong, Firestone et al 2002; Hong, Kim et al 2002). I3C has also been found to sensitise multidrug resistance (MDR) tumours to chemotherapeutic drugs without eliciting direct toxicity to the host (Christensen and LeBlanc 1996). Moreover, I3C may inhibit breast cancer invasion and migration (Meng, Goldberg et al 2000; Meng, Qi et al 2000). Because of these pleiotropic effects, the interest in I3C and DIM as cancer chemopreventive agents has increased significantly in the past years. Although dietary, epidemiological and experimental studies have shown the benefits of I3C and DIM in the prevention and inhibition of cancer, the molecular mechanism(s) by which I3C and DIM exert their tumour-suppressive effects on cancers have not been fully elucidated. This review summarises the well accepted inhibitory effects of I3C and DIM on cancer cells, and provides a comprehensive view on the molecular mechanism(s) of cancer chemopreventive and therapeutic effects of I3C and DIM.

Inhibition of oncogenesis

Oncogenesis is a multistep process, and there is a great opportunity for intervention to stop, revert or delay the oncogenic process. One anti-oncogenic action is the modulation of carcinogen metabolism, including inhibition of procarcinogen activation, induction of detoxification and blocking of reactive metabolites (Verhoeven et al 1997; van Iersel et al 1999). The molecules involved in this modulation are phase I and phase II biotransformation enzymes. I3C has been shown to inhibit chemically induced tumourigenesis of the liver, mammary gland, colon and other tissues, and suppress spontaneous carcinogenesis in mammary gland and endometrium (Bradlow et al 1991; Kojima et al 1994; Verhoeven et al 1997). It has been reported that treatment of rat with I3C increases CYP1A1, 1A2, 2B and 3A activity in rat liver (Stresser et al 1994; Manson et al 1998). I3C also up-regulates the level and activity of glutathione S-transferases (GST) (Bradfield and Bjeldanes 1984; Manson et al 1998). Therefore, the anti-oncogenic activity of I3C administered before or concurrently with a carcinogen is thought to be mediated through alternations in the levels and activities of phase I (eg p450 or CYP) and phase II (eg GST) isozymes in the liver and/or extrahepatic tissues, resulting in their increased capacity for detoxification of carcinogens.

The authors utilised cDNA microarray technique to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C and DIM. From microarray analysis, both I3C and DIM up-regulated the expression of phase I and II enzymes in PC3 prostate cancer cells (Li et al 2003). The most important phase I enzymes are cytochrome p450 enzymes, which oxidise carcinogens and make them more hydrophilic and susceptible to detoxification. Phase II metabolism comprises detoxification and conjugation reactions, making phase I metabolites more polar and readily excretable. I3C and DIM up-regulated

the expression of phase I enzyme cytochrome p450 1A1 (CYP1A1), phase II enzymes glutathione S-transferase theta 1 (GSTT1) and aldo-keto reductase (AKR) in PC3 prostate cancer cells, suggesting their increased capacity for detoxification and inhibition of carcinogens (Li et al 2003). These results are consistent with the experimental reports published by other investigators (Bradfield and Bjeldanes 1984; Vang et al 1990; Wortelboer et al 1992; Takahashi et al 1995; Lake et al 1998; Manson et al 1998; Katchamart and Williams 2001; Nho and Jeffery 2001; Horn et al 2002). They provide molecular evidence demonstrating that I3C and DIM may serve as a chemopreventive agent against prostate and other human cancers because of their ability to induce phase I and phase II enzymes.

From the cDNA microarray analysis, it was also found that DIM, but not I3C, induced the expression of other phase II enzymes, including methylmalonate-semialdehyde dehydrogenase, phospholipase A2, carbohydrate sulfotransferase 7 and glucuronosyltransferase I, indicating that DIM may have more of an inhibitory effect on oncogenesis compared to I3C (Li et al 2003). These results are very interesting and require further in-depth investigation (currently in progress).

It has been well known that oestrogen and androgen elicit their oncogenic actions in hormone-related cancers, such as breast, cervical, endometrium and prostate cancers. I3C has been shown to suppress tumourigenesis of oestrogen-responsive tissues (Bradlow et al 1991; Kojima et al 1994). Clinical trials also show that I3C at a minimum effective dose schedule is a promising chemopreventive agent for breast cancer prevention (Wong et al 1997). Studies from several laboratories have shown that I3C is a negative regulator of the oestrogen receptor (OR) signalling pathway (Riby et al 2000; Auborn et al 2003). By competitive binding to OR, I3C was found to inhibit the expression of genes driven by OR, resulting in cell growth inhibition and apoptosis. In addition, I3C has been shown to induce oestradiol metabolism through the induction of cytochrome p450 (CYP), resulting in a decrease in active oestrogen (Horn et al 2002). Clinical trials also show that I3C could be useful to increase the 2-OH-oestrone:oestriol metabolite ratio without detectable side effects in women (Bradlow et al 1994). Moreover, DIM binds to AhR (aryl hydrocarbon receptor), functions as an AhR agonist and down-regulates oestrogen-induced genes by blocking the crosstalk between AhR and OR (Chen I et al 1996; Chen et al 1998; Chen I et al 2001). A recent study shows that DIM is a strong androgen antagonist that blocks expression of androgen-responsive genes and inhibits androgen receptor nuclear translocation. This results in the inhibition of cell proliferation and DNA synthesis in LNCaP prostate cancer cells (Le et al 2003). These results demonstrate that I3C and DIM also exert their anti-oncogenic effects through modulation of OR, AR and AhR signalling pathways. However, it is important to note that I3C and DIM show inhibitory effects on both hormone-related and unrelated cancers, suggesting the comprehensive molecular mechanisms of their actions. Hence, the focus of this review is primarily based on recent molecular knowledge that may be applicable to cancer cells irrespective of their status of OR, AR, AhR or cytochrome p450.

Inhibition of cancer cell growth and induction of cell cycle arrest

It has been shown that I3C and DIM inhibit the growth of cancer cells including breast, prostate, colon and cervical cancer cells (Jin et al 1999; Bonnesen et al 2001; Chen DZ et al 2001; Hong, Firestone et al 2002; Hong, Kim et al 2002; Frydoonfar et al 2003; Kim et al 2003; Nachshon-Kedmi et al 2003). The authors have experimentally studied the effects of I3C and DIM on the growth of various cancer cells, including PC3 prostate cancer cells, MDA-MB-231, MDA-MB-435, and MCF10CA1a breast cancer cells (Rahman et al 2000; Chinni et al 2001; Li et al 2003; Rahman et al 2003). The growth of these cancer cells was inhibited by treatment with 30–100 μM I3C or 20–60 μM DIM for a period of 24–72 hours. The inhibition of cell growth was found to be dose- and time- dependent. These effects of I3C and DIM clearly indicate that they could be very useful for inhibiting the cell growth of various cancer cells.

The growth inhibition of cancer cells could be due to cell-cycle arrest, which ultimately results in ceasing cell proliferation. It has been reported that I3C can induce a G1 cell cycle arrest in breast cancer cells (Cover et al 1998; Hong, Kim et al 2002). Data also showed that I3C induced a G1 cell cycle arrest in PC3 prostate cancer cells (Chinni et al 2001). The induction of cell cycle arrest in breast and prostate cancer cells by I3C could be mediated through the regulation of expression of genes involved in the control of the cell cycle.

The progression of the cell cycle is known to be tightly regulated by different cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs)

in different phases of the cell cycle. The data showed a dose- and time-dependent decrease in the expression of CDK6 in I3C-treated PC3 prostate cancer cells (Chinni et al 2001). Moreover, I3C inhibited the CDK6 kinase activity, which plays important roles in the regulation of G1 phase progression (Chinni et al 2001). Other studies have also reported that I3C inhibited the expression of CDK6 in breast cancer cells (Cover et al 1998; Cram et al 2001). Cyclins/CDKs complex is needed for cells passing through the cell cycle; however, the activity of cyclins/CDKs complex is negatively regulated by several CDK inhibitors (CDKIs) including $p21^{WAF1}$, $p27^{KIP1}$ and p16. The authors examined whether I3C could alter the expression of $p21^{WAF1}$ and $p27^{KIP1}$ in PC3 prostate cancer cells by Western blot. The data revealed a significant dose-dependent up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$ expressions in I3C-treated cancer cells compared to control cells (Chinni et al 2001). This finding was consistent with the results on the inhibition of cancer cell growth, cell cycle arrest and down-regulation of CDK6, all of which demonstrate that I3C can inhibit the growth of cancer cells by modulating the expression of genes that are involved in the regulation of cell proliferation and cell cycle. The up-regulation of $p21^{WAF1}$ and $p27^{KIP1}$ and down-regulation of CDK6 may be one of the molecular mechanism(s) by which I3C inhibits cancer cell growth and induces cell cycle arrest (Figure 2).

Induction of apoptosis

In addition to cell cycle arrest, the overall cell growth inhibition induced by I3C could also be due to increased programme cell death known as apoptosis. To address this issue, apoptosis assays were conducted in MDA-MB-435 and MCF10CA1a breast cancer cells, and PC3 prostate cancer cells treated with I3C (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). For apoptosis assays, nucleosomal DNA ladders are widely used as biochemical markers of apoptosis. The cleavage of poly(ADP-ribose) polymerase (PARP) has also been used as early markers of apoptosis (Lazebnik et al 1994). Additionally, flow cytometric analysis with 7AAD staining has been conducted to detect and quantify apoptotic cells (Philpott et al 1996). By using these techniques, induced apoptosis in all cancer cells tested occured when they were treated with 60–100 μM of I3C. DNA ladder formation and PARP cleavage were observed in cancer cells treated with I3C for 48 hours (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). Flow cytometry revealed that the number of apoptotic cells increased up to 58%–84% with longer I3C treatment (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). These results clearly suggest that I3C can induce apoptosis, and are consistent with studies reported by other investigators (Ge et al 1996; Katdare et al 1998; Ge et al 1999; Bonnesen et al 2001; Chen DZ et al 2001; Hong, Firestone et al 2002; Zheng et al 2002; Nachshon-Kedmi et al 2003).

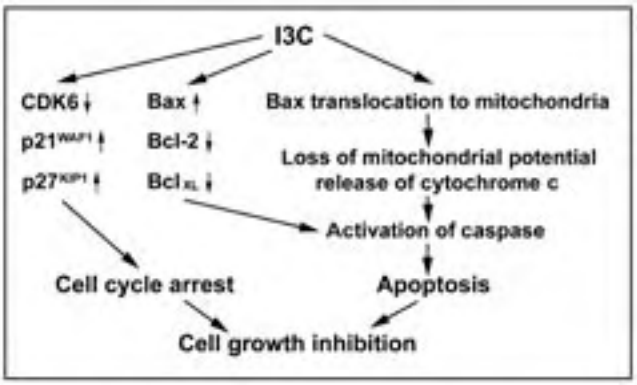

Figure 2 The effects of I3C on cell cycle arrest and apoptosis.

To exploit the molecular mechanism(s) of I3C-induced apoptosis, the authors investigated the alternation of gene expression involved in the apoptotic pathway. Bax, Bcl-2 and Bcl_{XL} have been reported to play a major role in determining whether cells will undergo apoptosis under experimental conditions that promote cell death (Kane et al 1993; Park and Hockenbery 1996; Findley et al 1997; Cory and Adams 2002). Increased expression of Bax could induce apoptosis, while Bcl-2 and Bcl_{XL} protect cells from apoptosis. It has also been found that the ratio of Bax:Bcl-2, rather than Bcl-2 alone, is important for the survival of drug-induced apoptosis (Salomons et al 1997). The data showed a reduced level in Bcl-2 and Bcl_{XL} protein expression in prostate and breast cancer cells tested after treatment with I3C for 48 hours and longer (Rahman et al 2000; Chinni et al 2001; Rahman et al 2003). The expression of Bax was up-regulated after I3C treatment for 24 hours. The ratio of Bax to Bcl-2 was significantly increased after 24 hours of treatment, corresponding with a significant increase in apoptotic cells after 48 hours of I3C treatment. A similar result was also observed in I3C-treated breast cancer cells by other investigators (Hong, Firestone et al 2002). These results suggest that up-regulation of Bax and down-regulation of Bcl-2 and Bcl_{XL} may be one of the molecular mechanism(s) by which I3C and DIM induces apoptosis (Figure 2).

Bax translocation from cytosol into mitochondria has been known as a critical event that occurs during

apoptotic processes (Bedner et al 2000; Jia et al 2001). By using immunostaining and confocal imaging techniques, the translocation of Bax from cytosol into mitochondria in both MCF10A non-tumourigenic and MCF10CA1a cancer cells treated with I3C (Rahman et al 2003) was observed. However, no significant apoptosis was seen in MCF10A non-tumourigenic cells treated with I3C. It has been reported that the translocation of Bax from cytosol into mitochondria targets the mitochondrial intermembrane contact sites. This causes mitochondrial permeability transition, loss of mitochondrial potential, release of cytochrome c, subsequent activation of caspases and DNA fragmentation resulting in apoptotic cell death (Putcha et al 1999; Gao et al 2001; De Giorgi et al 2002). I3C induces the loss of mitochondrial potential and the release of cytochrome c in MCF10CA1a breast cancer cells but not MCF10A non-tumourigenic cells. This occurs even though Bax was translocated from cytosol into mitochondria upon I3C treatment in both cells (Rahman et al 2003), suggesting that I3C-induced loss of mitochondrial potential is a more important and direct event for the release of cytochrome c and induction of apoptosis in cancer cells (Figure 2). The fact that I3C selectively induces the loss of mitochondrial potential, the release of cytochrome c and apoptosis in MCF10CA1a breast cancer cells rather than MCF10A non-tumourigenic cells, makes I3C an ideal agent for preventive and/or therapeutic purposes against breast and other cancers.

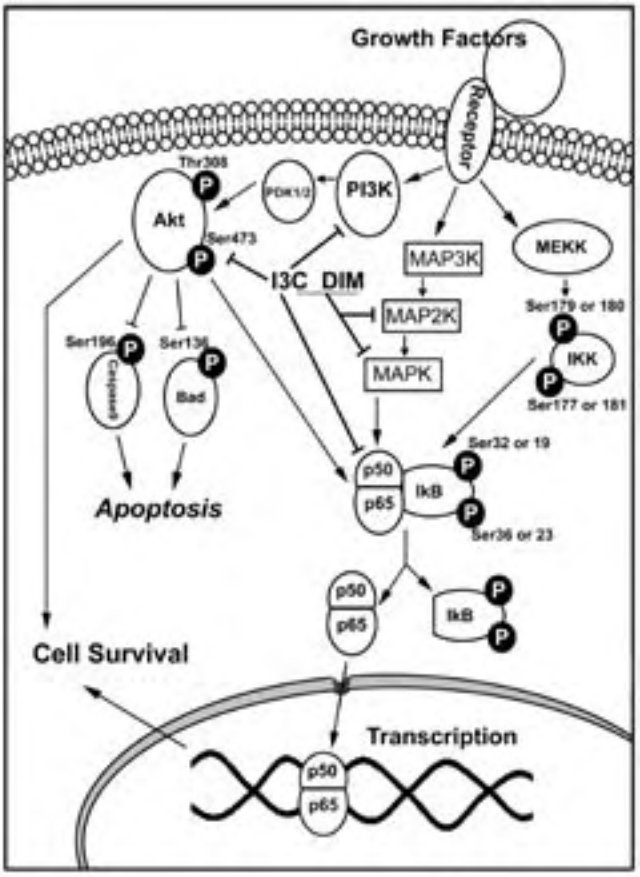

Figure 3 Akt, NF-κB and MAPK signalling pathways and the effects of I3C and DIM on those pathways.

Inhibition of NF-κB and Akt pathways

The nuclear factor-κB (NF-κB) pathway plays an important role in the control of cell growth, differentiation, apoptosis, inflammation, stress response and many other physiological processes in cellular signalling. The NF-κB family is composed of five proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52), each of which may form homo- or hetero-dimers (Ghosh et al 1995; Muller et al 1995; Verma et al 1995). In human cells, NF-κB is sequestered in the cytoplasm through tight association with its inhibitory protein, IκB. The activation of NF-κB occurs through site-specific phosphorylation and ubiquitination of IκB protein by IKK. IκB is subsequently degraded by the 26S proteasome (Chen et al 1995; Traenckner et al 1995; Chen ZJ et al 1996). This process allows NF-κB to become free from IκB and to translocate into the nucleus for binding to NF-κB-specific DNA-binding sites and, in turn, regulating gene transcription (Figure 3). Recent reports demonstrate that IKK-α also phosphorylates histone H3 and regulates the activation of NF-κB-directed gene expression (Anest et al 2003; Israel 2003; Yamamoto et al 2003). NF-κB controls the expression of many genes that are involved in the cellular and physiological processes as mentioned above. The disorder of these physiological processes has been demonstrated to link with the onset of cancers and chronic diseases. Therefore, NF-κB has been described as a major culprit and a therapeutic target in cancer (Biswas et al 2001; Bharti and Aggarwal 2002; Haefner 2002; Hideshima et al 2002; Orlowski and Baldwin 2002).

Because of the importance of NF-κB, as mentioned above, the authors investigated whether I3C treatment could modulate NF-κB DNA-binding activity in PC3 prostate cancer cells by electrophoresis mobility shift assay (EMSA) (Chinni et al 2001). PC3 cells were treated with 60 μM I3C for 48 hours. Nuclear extracts were harvested from control and I3C-treated cells, incubated in binding buffer with ^{32}P-labelled NF-κB consensus oligonucleotide, and

subjected to 8% non-denatured polyacrymide gel. After drying the gel, autoradiography of the gel revealed that 60 μM I3C significantly inhibited NF-κB DNA binding in PC3 prostate cancer cells.

The Akt signalling pathway is another important signal transduction pathway in human cells. Also referred to as PKB, Akt plays a critical role in controlling the balance between cell survival and apoptosis (Burgering and Coffer 1995). It contains an amino-terminal pleckstrin homology (PH) domain that binds phosphorylated lipids at the membrane in response to activation of PI3 kinases. Akt may be activated by insulin and various growth and survival factors through the activation of PI3 kinase (Franke et al 1995). It is activated by phospholipid binding and phosphorylation at Thr308 by PDK1, and also by phosphorylation within the C-terminus at Ser473 by PDK2 (Alessi et al 1996; Rommel et al 1999). Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets, including Bad, Forkhead transcription factors and caspase-9, all of which are involved in apoptotic pathways (Brunet et al 1999; Rommel et al 1999). Recent reports show that it also regulates the NF-κB pathway via phosphorylation and activation of molecules in the NF-κB pathway (Figure 3) (Ozes et al 1999; Romashkova and Makarov 1999). Akt has also been described as a target in cancer therapy (Chang et al 2003).

Because Akt pathways play an important role in cell growth, apoptosis and oncogenesis, the status of Akt in PC3 cells treated with 30, 60 and 100 μM I3C by immunoprecipitation, Western blot and kinase assays (Chinni and Sarkar 2002) were examined. No alterations on the total Akt protein expression in I3C-treated PC3 cells were found. However, a decrease in the phosphorylated Akt protein at Ser473 and Thr308 was seen in the I3C-treated PC3 cells compared to control cells, suggesting inactivation of Akt kinase after I3C treatment. These results were confirmed by Akt kinase assay, which showed a decrease in the Akt kinase activity in I3C-treated PC3 cells. Examining the Akt status in PC3 cells pretreated with I3C followed by EGF stimulation, it was found that EGF treatment alone activated Akt kinase as expected, while I3C pre-treatment abrogated the activation of Akt by EGF. These data demonstrate that I3C inhibits the activation of Akt and Akt kinase activity, which may result in the inhibition of survival signals and induce apoptotic signals. The inhibition of NF-κB and Akt pathways by I3C may be another molecular mechanism by which I3C induces apoptosis. The fact that I3C inhibits both NF-κB and Akt pathways, which are known as therapeutic targets for cancer therapy, makes I3C an ideal agent for preventive and/or therapeutic purposes against cancer.

Regulation of gene expression profiles

Although interest in the molecular effects of I3C and DIM on cancer cells has greatly increased in recent years, little is known about the global gene expression profiles of cancer cells after I3C or DIM treatment. The precise molecular mechanism(s) by which I3C and DIM exert their tumour suppressive effects is still unclear. To establish such molecular mechanism(s), the high-throughput gene chip, which contains 22 215 known genes, was utilised to determine the alternation of gene expression profiles of PC3 prostate cancer cells exposed to I3C or DIM (Li and Sarkar 2003). The results from cDNA microarrays provided a genome-wide analysis of the cellular response to I3C and DIM treatment. It was found that the alternation of biological processes and molecular functions in I3C- and DIM-treated PC3 cells are complex and are likely to be mediated by a variety of regulatory pathways (Li and Sarkar 2003). Thus, the global gene expression profile of cancer cells after I3C or DIM treatment opens new avenues to further investigate the molecular mechanism(s) by which I3C and DIM inhibit cancer cells.

Through using cDNA microarray analysis, it was found that the molecular response to both I3C and DIM in PC3 prostate cancer cells mainly involves the inhibition of genes that are related to cell proliferation, cell cycle control, apoptosis, signal transduction, oncogenesis and transcription regulation (Li and Sarkar 2003). EGFR, TGF-β2 and FGF play important roles in promoting cell growth and angiogenesis (Cross and Claesson-Welsh 2001; Platten et al 2001; Mendelsohn 2002). The down-regulation of EGFR, TGF-β2 and FGF by I3C or DIM treatment corresponded with the growth inhibitory effects of I3C and DIM. Cyclin E, activating transcription factor (ATF), and mitogen inducible gene (MIG) regulate cell cycle progression and apoptosis (Jean and Bar-Eli 2001; Nakayama et al 2001). The results also showed that I3C and DIM inhibited the expression of cyclin E2, ATF5, MIG-2 and Bcl-2, and induced the expression of $p57^{KIP2}$, which may lead to the induction of cell cycle arrest and apoptosis.

Cell signal transduction pathways are important for cell survival. In addition to NF-κB and Akt pathways targeted by I3C and DIM as mentioned earlier, MAPK has received much attention in cancer research and is believed to be a target for cancer prevention and therapy. The MAPK pathway consists of a three-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (Figure 3), resulting in the activation of NF-κB, cell growth and cell survival (Seger and Krebs 1995; Sebolt-Leopold 2000). Down-regulation in the expression of MAP2K3, MAP2K4, MAP4K3 and MAPK3 by I3C and DIM treatment was observed, suggesting the inhibitory effects of I3C and DIM on the MAPK pathway, which is likely to result in the inhibition of cancer cell survival. From the gene expression profiles of PC3 cells exposed to I3C, the down-regulation of PI3K expression was found to be consistent with results showing inactivation of the Akt kinase pathway by I3C.

Several Pol II transcription factors including TFDP and NF-YC play important roles in cell transcription (Yasui et al 2002; Romier et al 2003). TFDP1 overexpression leads to up-regulation of cyclin E, which encodes a positive regulator for cell cycle G1/S transition (Yasui et al 2002). Their overexpression has also been related to oncogenesis. In addition, core binding factor beta (CBF-β) and suppressor of tumourigenicity 16 (ST16) are also known to be involved in the processes of oncogenesis. CBF-β forms fusion protein with other gene product and promotes oncogenesis, while ST16 suppresses the oncogenesis (Jiang et al 1996; Kundu and Liu 2001). The results from gene expression profiling showed that I3C and DIM down-regulated the expression of TFDP1, NF-YC, cyclin E and CBF-β, and up-regulated ST16 expression. This suggests that I3C and DIM-mediated inhibition in transcription and oncogenesis involves multiple genes, all of which could be specific targets of I3C and DIM.

Conclusions

The studies presented in this review article clearly indicate that I3C and DIM, generally obtained from natural food sources, play important roles in cancer prevention and may perhaps be useful for treatment. These effects are believed to be mediated through the regulation of cell cycle, cell proliferation, apoptosis, oncogenesis, transcription and cell signal transduction. Therefore, the inactivation of Akt, NF-κB, MAPK and Bcl-2 signalling pathways may represent the molecular mechanism(s) by which I3C and DIM exert their anticancer effects. However, the biggest challenge is to demonstrate whether specific targets are affected by I3C/DIM in vivo. Therefore, further in vitro and in vivo investigations along with clinical trials are needed to fully appreciate the value of I3C and DIM in the fight against human cancers.

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alter microtubule structure in prostate and breast cancer cell lines (Rafi et al, 2002). In continuation of our earlier observations, we investigated the mechanism of action and signaling events in beta-hydroxy-DHP induced apoptosis of human colon cancer cells (RKO) in vitro. We have shown that beta-hydroxy-DHP induces the DNA fragmentation and PARP cleavage in RKO cells in a dose dependent manner. To elucidate the molecular events involved in its effect, we have demonstrated that there is significant induction of p53 and cell cycle inhibitory protein p27. Furthermore, the induction of proapoptotic protein Bax and phosphorylation of antiapoptotic protein Bcl-2 have also been observed. Surprisingly, this compound also induces the sustained activation of mitogen activated protein kinase (Erk1/2), but not found responsible for induction of apoptosis. We also tested the effect of protein kinase C (PKC) specific inhibitor (chelerythrine) and receptor tyrosine kinases (RTKs) specific inhibitors (Genistein) on beta-hydroxy-DHP induced apoptosis of RKO cells. However, none of the inhibitors are able to abrogate the beta-hydroxy-DHP induced DNA fragmentation, suggesting that PKC or RTK is not involved in beta-hydroxy-DHP induced apoptosis. Therefore, the involvement of other upstream kinases like mitogen activated protein kinase (p38) and stress activated protein kinase/cJun N-terminal kinase (SAPK/JNK) in beta-hydroxy-DHP induced apoptosis of RKO cells is currently under investigation.

#6548 Methioninase induces DNA hypomethylation in cancer cells. David Machover, Jacqueline Zittoun, Raphael Saffroy, Philippe Broet, Stephane Giraudier, Thierry Magnaldo, Emma Goldschmidt, Brigitte Debuire, Mireille Orrico, Yuying Tan, Zohar Mishal, Odile Chevallier, Carole Tonetti, Helene Jouault, Ayhan Ulusakarya, Marie-Laure Tanguy, Gerard Metzger, and Robert M. Hoffman. *Hospital Paul-Brousse, Villejuif, France, Hospital Henri-Mondor, Creteil, France, Institute Andre-Lwoff, Villejuif, France, and AntiCancer, Inc., San Diego, CA.*

DNA methylation is frequently altered in cancer, thereby affecting the expression of large numbers of genes. Promoter hypermethylation has been shown to silence potential tumor suppressor gene. One approach to reversing aspects of malignancy is to reactivate the suppressor genes. Toxic agents such as 5-azacytidine are currently available to perturb DNA mthylation in cells. In this report, we show that nontoxic levels of recombinant methioninase (rMETase) is as potent as 5-azacytidine in reducing DNA methylation in cancer cells. Human CCRF-CEM leukemia cells treated with rMETase contained significantly lower levels of genomic methylated DNA than did control cells. DNA hypomethylation was demonstrated by incorporation of the methyl radical of [*methyl*-3H]*S*-adenosylmethionine in DNA and by use of methylation-sensitive arbitrarily primed PCR. DNA hypomethylation produced by rMETase was of similar magnitude as that produced with the DNA methyltransferase inhibitor 5-azacytidine. Cells exposed to rMETase synthesized significantly more DNA than did untreated cells. Incorporation of [6-3^H]thymidine and [6-3^H]2-deoxyuridine in these cells was increased over that in control by mean factors of 1.78 and 2.36, respectively. Increased 3H-nucleoside incorporation resulted in greater numbers of nuclear grains as demonstrated by autoradiography. DNA hypomthylation and enhanced DNA synthesis appears to be a coordinated response. rMETase shows promise as a non-toxic effector of DNA hypomethylation in contrast to the toxic 5-azacytidine and will be tested for reactivation of methylation-silenced tumor suppressor genes.

#6549 Isodiospyrin, a novel agent that inhibits both DNA relaxation and kinase activities of human DNA topoisomerase I. Jaulang Hwang, Chun-Yuan Ting, and Chia-Tse Hsu. *Institute of Molecular Biology, College of Science, National Chung-Cheng University, Chia-Yi, Taiwan, Institute of Molecular Biology, Taipei, Taiwan, and Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.*

Isodiospyrin is a natural product of Diospyros morrisiana that consists of an unsymmetrical 1,2-binaphthoquinone chromophore. This compound has potent antitumor activity, yet very little was known about its cellular target and action mechanism. In the present study, we show that isodiospyrin is a novel agent capable of inhibiting both DNA relaxation and kinase activities of human DNA topoisomerase I. Isodiospyrin is neither a DNA intercalative agent and nor a DNA cleavage agent mediated by topoisomerase I. However, it blocks the action of camptothecin on DNA cleavage mediated by topoisomerase I. Furthermore, Isodiospyrin exhibits strong inhibitory effect on kinase activity of topoisomerase I toward SF2/ASF. This study demonstrates that isodiospyrin interacts with DNA topoisomerase I directly resulting in blocking both DNA and ATP binding to the enzyme and as a result affecting both DNA relaxation and kinase activity of topoisomerase I.

#6550 2-Chlorodeoxyadenosine: Mechanism of disruption of DNA: TATA element binding protein (TBP) interactions. Ted Foley, Patricia Hentosh, and D. Eric Walters. *Finch University of Health Sciences, N. Chicago, IL and The University of Health Sciences, Kansas City, MO.*

The nucleoside analog, 2-chlorodeoxyadenosine (CldAdo, cladribine), is highly effective as a primary agent for hairy cell leukemia and chronic lymphocytic leukemia. Previously we showed that CldAMP residues within a promoter TATA element interfered with eukaryotic transcriptional machinery by affecting TATA box binding protein (TBP) binding, stability and functionality. To understand such effects, we used molecular simulation to model the TBP core domain complexed to a CldAMP-substituted Adenovirus major late promoter TATA element ([5′-TATAAAAG numbered 5-12 for the coding strand] and [3′-ATATTTTC numbered 112-105 for the complementary strand]). The x-ray crystal structure (1.90 Å resolution) of the TBP:TATA complex was downloaded from the Protein Data Bank (listed as 1CDW). This structure was modeled using Quanta software from Molecular Simulations Inc. with energy calculations via the CHARMM force field. Each of seven dAMP residues in the consensus sequence was replaced individually by CldAMP and the complex was minimized for 1200 steps. Conformational changes were measured by calculating root mean square (RMS) values for the substituted TBP structure relative to an unsubstituted complex. Compared to control, CldAMP within the TBP:TATA complex induced conformational changes in the protein as well as the DNA during minimization. RMS values for TBP showed the most extensive conformational change by a CldAMP-8 substitution (RMS=3.63) followed by CldAMP-6 (RMS=1.74), while CldAMP-10 had the least (RMS=0.78). All substitutions except CldAMP-110 resulted in overlapping van der Waal radii of the chlorine atom with various TBP amino acids, and DNA base-pairing hydrogen bonds were extensively disrupted. CldAMP-110 had the most dramatic effect on the distance between the phosphate backbones in the minor groove and produced the largest change in roll angle. These results indicate that the size and electronegative nature of the chlorine moiety in deoxyadenosine appreciably alter protein:DNA interactions, with each substituted site inducing unique modifications to TBP and/or DNA. Supported by NIH CA55414.

#6551 Gene expression profiles of I3C and DIM treated PC3 prostate cancer cells by cDNA microarray analysis. Yiwei Li, Xingli Li, and Fazlul H. Sarkar. *Wayne State University School of Medicine, Detroit, MI.*

Studies from our laboratory and others have shown that Indole-3-carbinol (I3C) and its in vivo dimeric product 3,3-diindolylmethane (DIM) inhibit the growth of PC3 prostate cancer cells and induce apoptosis by inhibiting NF-kB and Akt pathways. In order to obtain a comprehensive gene expression profiles altered by I3C and DIM treatment of PC3 cells, we have utilized cDNA microarray to interrogate 22,215 known genes by using Affymetrix Human Genome U133A Array. The total RNA from PC3 cells untreated and treated with 60μM I3C or 40μM DIM for 6 to 48 hours was subjected to microarray analysis and the data were analyzed using Microarray Suite and Data Mining, Cluster and TreeView, and Onto-express software. We found a total of 738 genes which showed >2 fold change after 24 hours of DIM treatment. Among these genes, 677 genes were down regulated and 61 genes were up-regulated. Similarly, 727 genes showed >2 fold change in the expression with down-regulation of 685 genes and up-regulation of 42 genes in I3C treated cells. The altered expressions of genes were observed as early as 6 hours and were significantly more evident with longer treatment. Upon cluster analysis, we found that both I3C and DIM up-regulated expression of genes that are related to the Phase I and Phase II enzymes (p4501A1, GSTT1, etc), suggesting their increased capacity for detoxification of carcinogens or chemicals. We also found that I3C and DIM down regulated the expression of genes, which are critically involved in the regulation of cell growth (EGFR, TGFβ2, IGFBP4, FGF, etc), cell cycle (cyclin E2, ATF5, Mig-2, etc), apoptosis (BCL2A1, etc), signal transduction (PI3K, MAPK, MAP2K3, MAP2K4, MAP4K3, etc), Pol II transcription factor (TFDP1, NF-YC, DKC1, etc), and oncogenesis (CBFB, ST16, etc). However, I3C and DIM also showed some differential effect on gene expression profile, which will be the subject of further study in our laboratory. In order to confirm our microarray data, we have also conducted Real-time RT-PCR analysis of selected genes. The results of Real-time RT-PCR and cDNA microarray data showed highest concordance. From these results, we conclude that I3C and DIM caused changes in the expression of a large number of genes that are related to the control of carcinogenesis, cell survival, and physiological behaviors. This may provide molecular mechanism(s) by which I3C and DIM exert their pleiotropic effects on PC3 prostate cancer cells and these information could be further exploited for devising chemopreventive and/or therapeutic strategies for prostate cancer.

#6552 Development of small molecules that antagonize IL-4/IL-13 mediated cell survival and enhance interferon activity in leukemia and lymphoma cells. Chun-Hong Xia, Sylvie Barchechath, Howard B. Cottam, Jongdae Lee, Christina C. Niemeyer, Halina Witkiewicz, Jack Reifert, James O. Tolley, Deirdre A. Trainor, Augusto F. Lois, Lorenzo M. Leoni, and Dennis A. Carson. *University California San Diego, La Jolla, CA, Salmedix, Inc, San Diego, CA, and Chromagen, Inc, San Diego, CA.*

Interleukin-4 (IL-4) and interleukin-13 (IL-13) mediate B-cell survival by protecting cells from apoptosis. IL-4 and IL-13 receptors are over-expressed in various

International Research Conference on Food, Nutrition, and Cancer

Indole-3-Carbinol and Prostate Cancer[1,2]

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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 κB (NF-κB), 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-κB 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),[4] 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

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[4] 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, IκB kinase; MIG, mitogen inducible gene; MAPK, mitogen-activated protein kinase; NF-κB, Nuclear factor κB; 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 μmol/L and DIM at 30 μmol/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 $p21^{WAF1}$ and $p27^{KIP1}$ in a dose-dependent manner (29). We immunoprecipitated cyclin D1 and cyclin E complexes, and detected CDK6, $p21^{WAF1}$, and $p27^{KIP1}$ in the complexes. The results showed that I3C decreased CDK6 binding to cyclin D1 and increased $p21^{WAF1}$ and $p27^{KIP1}$ 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 $p21^{WAF1}$ and $p27^{KIP1}$ 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**).

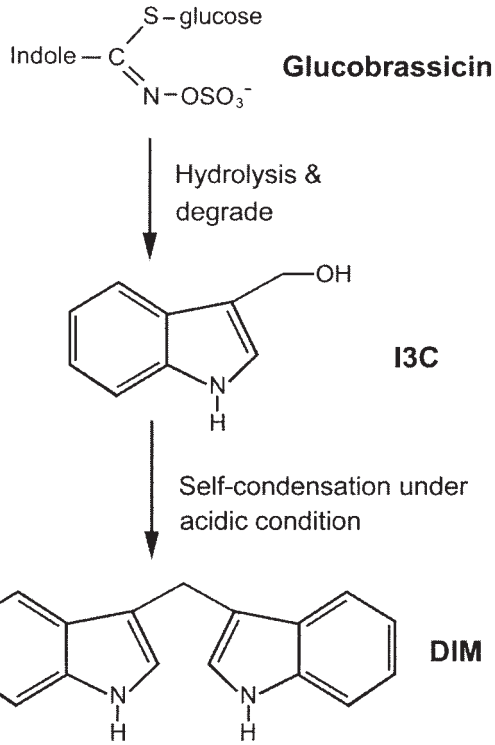

FIGURE 1 Molecular structure and metabolism of I3C and DIM.

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 μmol/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 μmol/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_{XL} play important roles in determining whether cells will undergo apoptosis (35,36). Bax promotes apoptotic cell death, whereas Bcl-2 and Bcl_{XL} 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_{XL} protein expression in PC3 prostate cancer cells treated with I3C at 60 μmol/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-

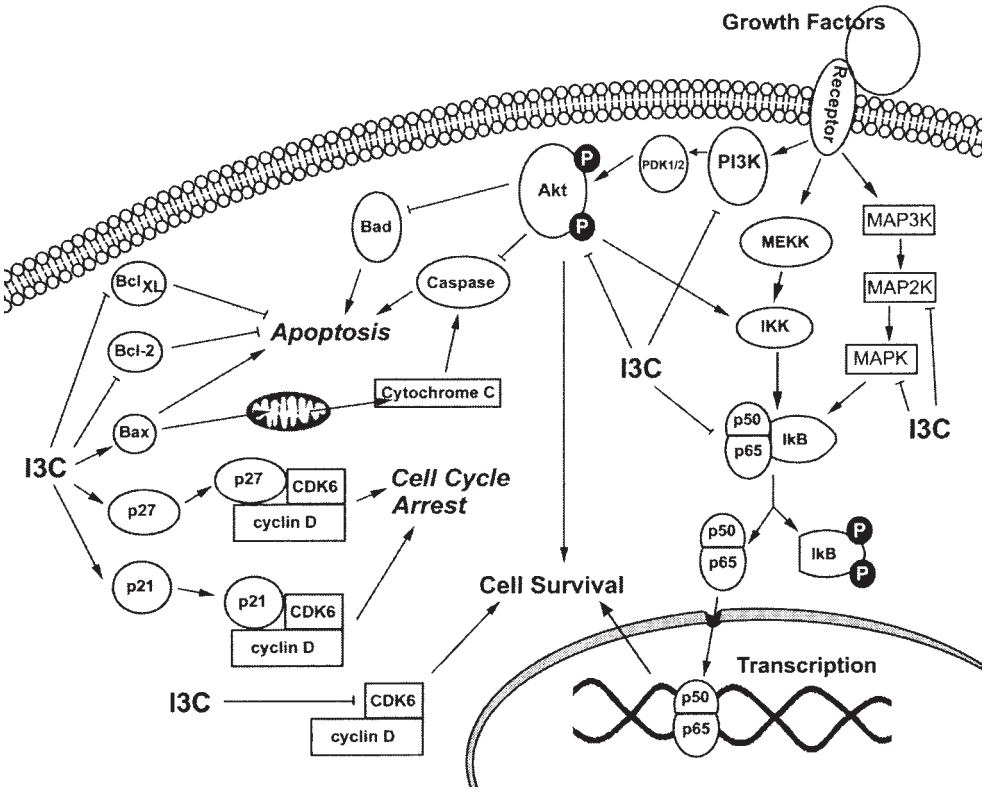

FIGURE 2 The effects of I3C on cell cycle, apoptosis, NF-κB, Akt, and MAPK pathways.

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_{XL} 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 κB pathway in prostate cancer cells

The nuclear factor κB (NF-κB) pathway plays an important role in many physiological processes in cellular signaling (43–45). In human cells, NF-κB is sequestered in the cytoplasm through tight association with its inhibitory protein, IκB. The activation of NF-κB occurs through site-specific phosphorylation of IκB by IκB kinase (IKK). IκB is subsequently degraded by the 26S proteasome. NF-κB becomes free from IκB and translocates into the nucleus for binding to NF-κB–specific DNA-binding sites, regulating target gene transcription. IKK-α also phosphorylates histone H3 and regulates the activation of NF-κB–directed gene expression. NF-κB 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-κB 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-κB has been described as a major culprit and a therapeutic target in cancer (43,46,47). Inhibition of NF-κB 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-κB DNA binding activity in PC3 prostate cancer cells (29). PC3 cells were treated with I3C at 60 μmol/L for 48 h or TNF-α at 50 μg/L for 15 min. Nuclear extracts were harvested from samples, incubated in binding buffer with ^{32}P-labeled NF-κB consensus oligonucleotide, and subjected to 8% nondenatured polyacrylamide gel. Autoradiography of the dried gel showed that TNF-α treatment stimulated NF-κB activation as expected; however, I3C at 60 μmol/L significantly inhibited NF-κB

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-κB 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-κB pathway through phosphorylation of molecules in the NF-κB 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 μmol/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_{XL} 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-κB (52). The activation of Akt and NF-κB 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-κB or Akt can potentiate the anticancer effect of chemotherapeutic agents (53,54). We found that I3C inhibited the activation of Akt and NF-κB 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 treatment with I3C and cisplatin significantly inhibited the growth of PC-3 prostate cancer cells compared with monotreatment (**Fig. 3**). These results suggest that I3C and DIM can potentiate the anticancer effect of chemotherapeutic agents through inactivation of Akt and NF-κB.

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,

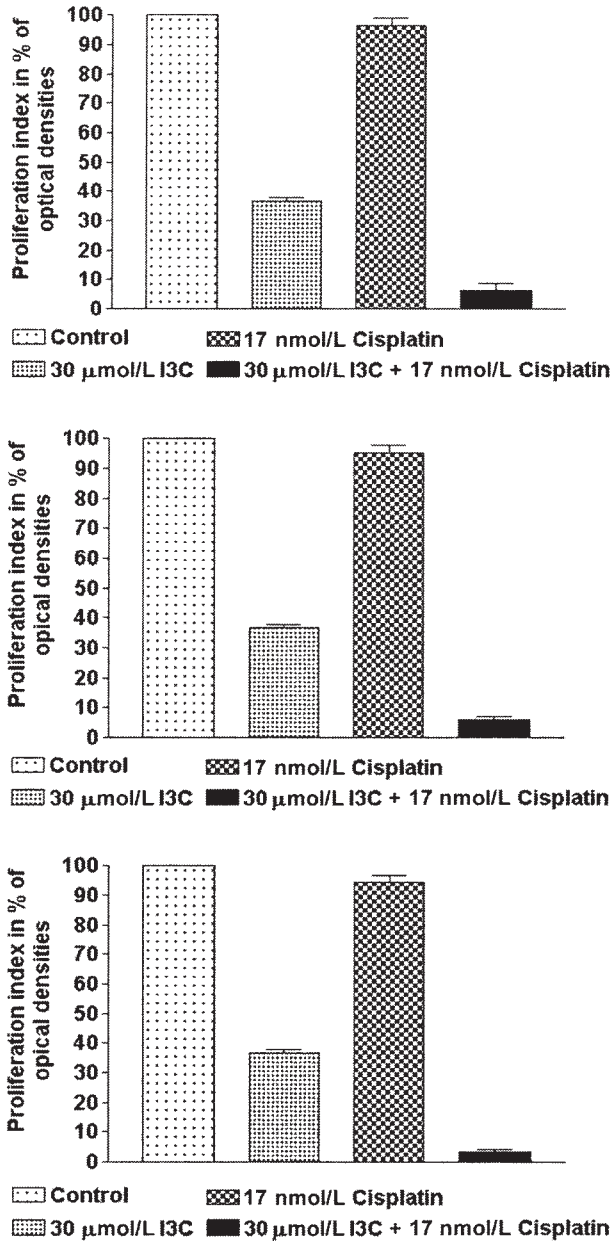

FIGURE 3 Growth inhibition of PC-3 prostate cancer cells exposed to I3C and cisplatin for 72 h (repeated 3 times).

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-β, 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 $p57^{KIP2}$, 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-κB 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-κB, 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-κB 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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Review

Cell signaling pathways altered by natural chemopreventive agents

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Abstract

Epidemiological studies have indicated a significant difference in the incidence of cancers among ethnic groups, who have different lifestyles and have been exposed to different environmental factors. It has been estimated that more than two-thirds of human cancers, which are contributed by mutations in multiple genes, could be prevented by modification of lifestyle including dietary modification. The consumption of fruits, soybean and vegetables has been associated with reduced risk of several types of cancers. The in vitro and in vivo studies have demonstrated that some dietary components such as isoflavones, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), curcumin, (−)-epigallocatechin-3-gallate (EGCG), apigenin, etc., have shown inhibitory effects on human and animal cancers, suggesting that they may serve as chemopreventive agents. Experimental studies have also revealed that these components regulate the molecules in the cell signal transduction pathways including NF-κB, Akt, MAPK, p53, AR, and ER pathways. By modulating cell signaling pathways, these components, among other mechanisms, activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibition of cancer development and/or progression. This article reviews current studies regarding the effects of natural chemopreventive agents on cancer-related cell signaling pathways and provides comprehensive knowledge of the biological and molecular roles of chemopreventive agents in cancer cells.

Keywords: Signaling pathway; Cancer; Prevention

Contents

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1. Introduction

It has been known that most human cancers are induced by environmental factors including chemical, radioactive and biological factors that exist in our living environment. There are significant differences in the cancer incidence and mortality among ethnic groups, who have different lifestyles and have been exposed to different environmental factors [1]. It has been estimated that more than two-thirds of human cancers could be prevented by modification of lifestyle including dietary modification [2]. The consumption of fruits, soybean and vegetables has been associated with reduced risk of several types of cancers [3–5]. The experimental in vitro and in vivo studies have demonstrated that some dietary components such as isoflavones, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), curcumin, (−)-epigallocatechin-3-gallate (EGCG), apigenin, have inhibitory effects on human cancers [6–13] suggesting that they may serve as chemopreventive agents.

The dietary components as chemopreventive agents have received much attention among the public and the medical community. Soy isoflavones including genistein, daidzein, and glycitein, mainly derived from soybean have been found to inhibit cancer growth in vivo and in vitro [14–16]. I3C and its in vivo dimeric product DIM, produced from naturally occurring glucosinolates in the family Cruciferae, have shown the inhibition of cancer cell growth through the modulation of genes that are related to the control of cell proliferation, cell cycle, apoptosis, signal transduction, oncogenesis, and transcription regulation [8,9]. Curcumin, a natural compound present in turmeric and possessing both anti-inflammatory and antioxidant effects, has been studied as a chemopreventive agent in several cancer models [11,17]. Beneficial effects attributed to green tea, such as its anticancer and antioxidant properties, are believed to be mainly mediated by EGCG [4,18]. Apigenin, one of the flavonoids, is widely distributed in many fruits and vegetables, and has been shown to possess anti-inflammatory and anticancer effects [19,20]. It is becoming clear that these dietary components exert their pleiotropic effects on cancer cells, affecting cell survival and physiological behaviors. However, the precise molecular mechanisms of actions of these components have not been fully elucidated, although the data from published literature does indicate that these components regulate transcription, translation and activation of the molecules in the cell signal transduction pathways. Among the cell signaling pathways, NF-κB, Akt, MAPK, p53, AR, and ER pathways are more important signaling pathways related to cancer development and progression. This article reviews current studies regarding the effects of natural chemopreventive agents on these cancer-related cell signaling pathways and provides comprehensive knowledge of the biological and molecular roles of chemopreventive agents in cancer cells.

2. Effects of chemopreventive agents on NF-κB

It has been well accepted that nuclear factor-κB (NF-κB) signaling pathway plays important roles in the control of cell growth, apoptosis, inflammation, stress response, and many other physiological processes [21–25]. There are several important molecules such as NF-κB, IκB, IKK, within NF-κB signaling pathway (Fig. 1). However, NF-κB is the key protein

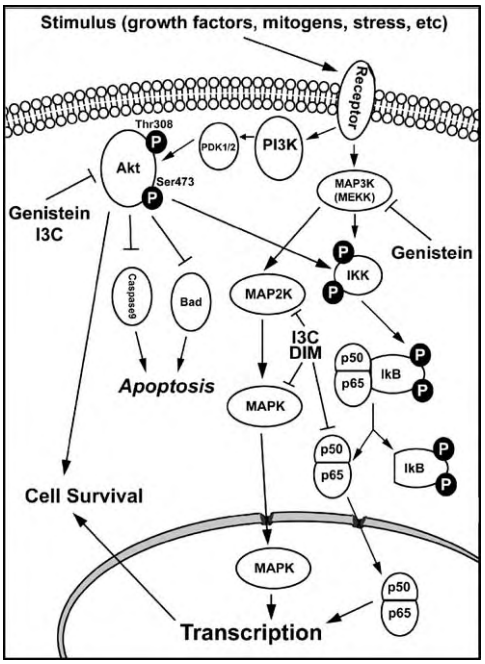

Fig. 1. Akt, NF-κB, and MAPK signaling pathways and the effects of genistein, I3C, and DIM on the pathways.

in the pathway, and has been described as a major culprit and a therapeutic target in cancer [26–29]. The data from experimental studies have demonstrated that genistein, I3C, curcumin, EGCG, and apigenin inhibit activation of NF-κB in different cancer cell lines, suggesting the inhibitory effects of these agents on cancer cells [8,20,30–33].

Our laboratory examined NF-κB DNA-binding activity in genistein treated PC3 and LNCaP prostate cancer cells by electrophoresis mobility shift assay (EMSA) [31]. The results showed that 50 μM genistein significantly inhibited the NF-κB DNA-binding activity in both cell lines. Furthermore, genistein pretreatment also abrogated the activation of NF-κB stimulated by H_2O_2 or TNF-α. Immunochemistry and confocal microscopic analysis also showed that TNF-α treatment significantly increased nuclear staining of the NF-κB p50 and p65 subunits, however, 24 h pretreatment of cells with genistein prior to TNF-α stimulation blocked p50 and p65 nuclear translocation. These results clearly demonstrate that genistein inhibits the translocation of NF-κB subunits to the nucleus, suggesting that genistein may reduce the NF-κB binding to its target DNA and thereby inhibit the transcription of target gene. Similar results in human lung epithelial cells and myeloid cells have been reported by other investigators [34,35]. It is important to note that the concentration of genistein used in experimental study is achievable in humans. The study reported by Busby et al. has shown that up to 27.46 ± 15.38 μM of genistein in human plasma can be achieved after receiving genistein supplement at a dose of 16.0 mg/kg [36], suggesting the bioavailability of genistein from supplement.

It has been found that oxidative stress activates NF-κB DNA binding activity [37,38]. Because soy isoflavones have been known as antioxidants, their inhibitory effects on oxidative stress may be mediated through inhibition of NF-κB DNA binding activity. We investigated whether soy isoflavone supplementation could inactivate NF-κB in vivo and reduce oxidative damage in lymphocytes in human volunteers [39]. We found that when human volunteers received 50 mg of soy isoflavone supplements Novasoy™ (Archer Daniels Midland Company, Decatur, IL, USA; containing genistein, daidzein, and glycitein at a 1.3:1:0.3 ratio) twice daily for 3 weeks, TNF-α failed to activate NF-κB activity in lymphocytes harvested from these volunteers, while lymphocytes from these volunteers collected prior to soy isoflavone intervention showed activation of NF-κB DNA binding activity upon TNF-α treatment in vitro. These results demonstrated that soy isoflavone supplementation had a protective effect against TNF-α induced NF-κB activation in humans. We further measured the levels of 5-OHmdU, a modified DNA base that represents endogenous status of cellular oxidative stress, in the peripheral blood lymphocytes of human volunteers before and after supplementation with Novasoy™. The mean value of 5-OHmdU before supplementation was 156.7 ± 25.72 and it was decreased to 60.83 ± 12.61 ($P < 0.01$) after 3 weeks of soy supplementation. These results provide evidence showing that soy isoflavones function as antioxidants and inhibit NF-κB activation, suggesting that these effects of soy isoflavones may be responsible for its cancer chemopreventive activity.

It has been known that NF-κB could be activated by phosphorylation and degradation of IκB [40]. IκB could be phosphorylated by activated IκB kinase (IKK), and IKK could be phosphorylated and activated by mitogen activated kinase kinase 1 (MEKK1), one of the molecules in MAPK pathway [41–43]. We found

that genistein treatment reduced the amount of phosphorylated IκB, demonstrating that genistein inhibits the phosphorylation of IκB and ultimately prevents the translocation of NF-κB to the nucleus. Moreover, we found that genistein treatment did not alter the protein expression of MEKK1; however, it did inhibit MEKK1 kinase activity in prostate cancer cells. These results suggested that genistein could inhibit MEKK1 kinase activity, which could be responsible for the decreased phosphorylation of IκB and, thereby, result in the inactivation of NF-κB (Fig. 1).

Our laboratory also investigated whether I3C treatment could modulate NF-κB DNA binding activity in PC3 prostate cancer cells by EMSA [8]. The results showed that 60 μM I3C significantly inhibited NF-κB DNA binding activity with induction of apoptosis in PC3 prostate cancer cells, suggesting that inhibition of NF-κB signaling pathway may be one of the molecular mechanisms by which I3C induces apoptosis in cancer cells (Fig. 1).

Other chemopreventive agents also show their inhibitory effects on NF-κB pathway. It has been reported that curcumin inhibited IKK, suppressed both constitutive and inducible NF-κB activation, and potentiated TNF-induced apoptosis [30]. Curcumin also showed strong antioxidant and anticancer properties through regulating the expression of genes that require the activation of activator protein 1 (AP1) and NF-κB [44]. It has been reported that EGCG treatment resulted in a significant dose- and time-dependent inhibition of activation and translocation of NF-κB to the nucleus by suppressing the degradation of IκBα in the cytoplasm [45,46]. EGCG also showed to inhibit activation of IKK and phosphorylation of IκBα [32,47]. It has been found that EGCG had a concurrent effect on two important transcription factors p53 (stabilization of p53) and NF-κB (negative regulation of NF-κB activity), causing a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis [13]. Apigenin treatment also resulted in down-modulation of the constitutive expression of NF-κB/p65 [33].

3. Effects of chemopreventive agents on Akt

Akt plays critical roles in mammalian cell survival signaling and has been shown to be activated in various cancers [48,49]. It has been known that Akt is activated by phospholipid binding and phosphorylation at Thr308 by PDK1 or at Ser473 by PDK2 [50]. Activated Akt functions to promote cell survival by inhibiting apoptosis through inactivation of several pro-apoptotic factors including Bad, Forkhead transcription factors, and caspase-9 [51–53]. Recent studies have also shown that Akt regulates the NF-κB pathway via phosphorylation and activation of molecules in the NF-κB pathway [54,55] (Fig. 1). Like NF-κB, Akt has also been believed to be an attractive target for cancer prevention or treatment [56].

The data from our laboratory showed that genistein inhibit both Akt and NF-κB pathways [57]. We found no alteration on the total Akt protein expression in genistein treated PC3 cells; however, decreases in the phosphorylated Akt protein at Ser473 and the Akt kinase activity were observed in genistein-treated PC3 cells, suggesting the inactivation of Akt after genistein treatment. We also found that genistein pretreatment abrogated the activation of Akt by EGF. To further explore the inhibitory mechanism of genistein on Akt and NF-κB pathways, Akt expression construct (pLNCX-Akt) was transiently co-transfected with NF-κB-Luc reporter construct into PC3 prostate cancer cells. Luciferase assay showed an increased luciferase activity in PC3 cells co-transfected with pLNCX-Akt and NF-κB-Luc. However, genistein inhibited the luciferase activity in PC3 cells co-transfected with pLNCX-Akt and NF-κB-Luc, and abrogated the activation in PC3 cells co-transfected with pLNCX-Akt and NF-κB-Luc followed by EGF stimulation. These results were further confirmed by examining NF-κB DNA-binding activity in transfected cells using EMSA, which showed similar results to those of transfection and luciferase assay. We also observed similar results in MDA-MB-231 breast cancer cells [58]. These results suggest that genistein exerts its inhibitory effects on NF-κB pathway through Akt pathway, and that down-regulation of NF-κB and Akt signaling pathways by genistein may be one of the molecular mechanisms by which genistein inhibits cancer cell growth and induces apoptosis (Fig. 1).

To explore the effect of I3C on Akt pathway, we examined Akt status in PC3 cells treated with 30, 60, and 100 μM I3C by Western blot, immunoprecipitation, and kinase assays [59]. We found a decrease in the phosphorylated Akt protein at Ser473 and Thr308 in I3C treated PC3 cells, suggesting inactivation of Akt after I3C treatment. These results were confirmed by

Akt kinase assay, which showed a decrease in the Akt kinase activity in I3C treated PC3 cells. We also found that I3C pretreatment abrogated the activation of Akt by EGF. From the gene expression profiles of PC3 cells exposed to I3C, we found down-regulation of PI3K expression, consistent with our results showing inactivation of Akt kinase by I3C [9]. These data demonstrated that I3C inhibited Akt signaling pathway, which may result in the inhibition of survival signals and the induction of apoptotic signals (Fig. 1).

Similar to our results about the effect of genistein on Akt, Kumar et al. have reported that an analogue of curcumin, 4-hydroxy-3-methoxybenzoic acid methyl ester (HMBME), targeted the Akt signaling pathway, inhibited the proliferation of human and mouse prostate cancer cells and induced apoptosis [60]. Transfection experiment showed that overexpression of constitutively active Akt reversed the HMBME-induced growth inhibition and apoptosis, demonstrating the direct role of Akt signaling in HMBME-mediated growth inhibition and apoptosis. HMBME also decreased the level of phosphorylated Akt, inhibited Akt kinase activity, and reduced DNA-binding activity of NF-κB [60]. Several reports by other investigators also suggest that curcumin has molecular targets within the Akt signaling pathways, and the inhibition of Akt activity may facilitate inhibition of proliferation and induction of apoptosis in cancer cells [61,62].

Recent report has shown that EGCG from green tea inhibits VEGF-induced angiogenesis in vitro through suppression of VE-cadherin phosphorylation and inactivation of Akt molecule, suggesting inhibitory effect of EGCG on Akt signaling pathway [63]. Masuda et al. also found that treatment with EGCG inhibited the constitutive activation of the Akt, EGFR, and Stat3 in both YCU-H891 head and neck squamous cell carcinoma and MDA-MB-231 breast carcinoma cell lines [64].

4. Effects of chemopreventive agents on MAPK

In addition to NF-κB and Akt pathways, MAPK has received increasing attention as a target molecule for cancer prevention and therapy. It has been reported that activation of the MAPK pathways may cause the induction of phase II detoxifying enzymes, and inhibition of MAPK pathways may inhibit AP-1-mediated gene expression [65]. MAPK pathway consists of a three-tiered kinase core where a MAP3K activates a MAP2K that activates a MAPK (ERK, JNK, and p38), resulting in the activation of NF-κB, cell growth, and cell survival [66,67] (Fig. 1).

We have utilized 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 [9]. From microarray data, we observed down-regulation in the expression of MAP2K3, MAP2K4, MAP4K3, and MAPK3 by I3C and DIM treatment, suggesting the inhibitory effects of I3C and DIM on MAPK pathway. The down-regulation of the important molecules in MAPK pathway may result in the inhibition of cancer cell survival (Fig. 1).

The ability of curcumin to modulate MAPK signaling pathway might contribute to the inhibition of inflammation by curcumin. Salh et al. reported that curcumin is able to attenuate experimental colitis through a reduction in the activity of p38 MAPK [68].

The reported effects of EGCG on MAPK pathway are controversial. EGCG showed strong inhibition of tyrosine kinase and MAPK activities in transformed NIH-pATM ras fibroblasts, without affecting the kinases in the normal cells [69]. Katiyar et al. reported that treatment of H_2O_2 resulted in phosphorylation of ERK1/2, JNK, and p38 in human epidermal keratinocytes [70]. When these cells were pretreated with EGCG, H_2O_2-induced phosphorylation of ERK1/2, JNK, and p38 was found to be significantly inhibited. These findings demonstrate that EGCG has the potential to inhibit oxidative stress-mediated phosphorylation of MAPK signaling pathways. Maeda-Yamamoto et al. also reported that EGCG inhibited the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), and suppressed p38 MAPK activity in human fibrosarcoma HT1080 cells [71]. However, EGCG has also been found to activate all three MAPKs (ERK, JNK and p38) in a dose- and time-dependent manner in human hepatoma HepG2-C8 cells [72]. In breast cancer cell line T47D, catechin (containing approximately 53% of EGCG) showed to phosphorylate JNK/SAPK and p38. The phosphorylated JNK/SAPK and p38 inhibited the phosphorylation of cdc2, and regulated the expression of cyclin A, cyclin B1, and cdk proteins, thereby causing G2 arrest [73]. It is possible that activation of MAPK by low concentration of EGCG re-

sults in induction of ARE-mediated gene expression, whereas higher concentration of EGCG causes activation of MAPKs such as JNK leading to apoptosis [72].

5. Effects of chemopreventive agents on p53

p53 is a tumor suppressor and transcription factor. It is a critical regulator in many cellular processes including cell signal transduction, cellular response to DNA-damage, genomic stability, cell cycle control, and apoptosis. As a tumor suppressor, functional p53 activates the transcription of downstream genes such as $p21^{WAF1}$ and Bax to induce the apoptotic process, inhibiting the growth of DNA damaged cells or cancer cells [74–76]. The status of p53 is thought to be an important mediator in the cellular response to chemotherapy [77].

In order to investigate whether genistein inhibits proliferation and induces apoptosis through p53 pathway in non-small cell lung cancer (NSCLC) cells, we measured cell growth inhibition, apoptosis, and gene expression related to apoptosis in genistein treated H460 cells, which harbor wild type p53, and H322 cells that possess a mutation in the p53 gene (codon 248, CGG to CTG, Arg to Leu) [78,79]. Genistein was found to inhibit both H460 and H322 cell growth in a dose-dependent manner. DAPI staining, poly(ADP-ribose) polymerase (PARP) cleavage, and flow cytometric apoptosis analysis showed that genistein at 30 μM caused cell death via a typical apoptotic pathway in both cell lines. Western blot analysis revealed that the expression of Bax and $p21^{WAF1}$ was up-regulated in both H460 and H322 cells treated with genistein. More importantly, significant up-regulation of p53 was detected in genistein-treated H460 cells, while no change in p53 expression was observed in H322 cells that underwent the same genistein treatment. Theses results suggest that genistein induces apoptosis in NSCLC cells through p53 independent pathway and, thus, may act as an anticancer agent regardless of the status of p53 in cancer cells.

Similar results have been observed in prostate cancer cells treated with EGCG. By using Western blot analysis, Gupta et al. found that EGCG treatment resulted in a dose-dependent increase of p53 in LNCaP cells (carrying wild-type p53), but not in DU145 cells (carrying mutant p53) [80]. They also found that EGCG induced stabilization of p53, which caused an up-regulation in its transcriptional activity, thereby resulting in the activation of its downstream targets such as $p21^{WAF1}$ and Bax and the induction of apoptosis. In a human liver cancer cell line, EGCG also significantly increased the expression of p53 and $p21^{WAF1}$ protein, and this contributed to cell cycle arrest [81].

Several studies examined the potential effects of I3C and DIM on the proliferation and induction of apoptosis in human prostate cancer cell lines with different p53 status. They found that induction of apoptosis by I3C was p53-independent [82]. Also, induction of $p21^{WAF1}$ expression by DIM was independent of estrogen-receptor signaling and p53 [83].

6. Effects of chemopreventive agents on AR

It has been found that androgen receptor (AR) signaling pathway plays important roles in the carcinogenesis and cancer progression through regulation of transcription of androgen-responsive genes [84] (Fig. 2). Several chemopreventive agents including genistein, I3C, DIM, and curcumin, have been found

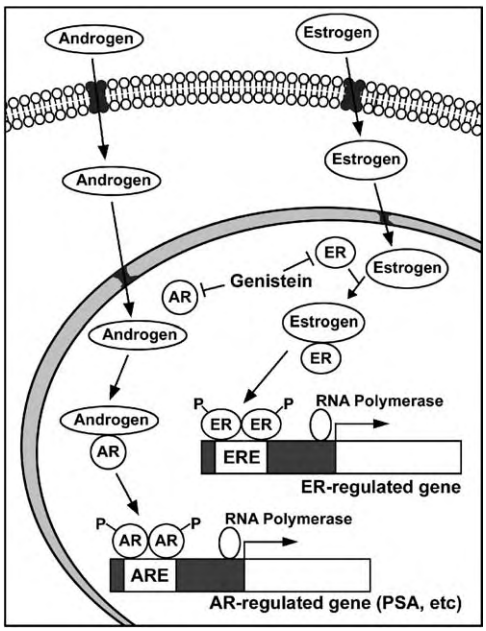

Fig. 2. AR and ER signaling pathways and the effects of genistein on the pathways.

to regulate the molecules in AR signaling pathway when they were used to inhibit growth of cancer cells.

Prostate specific antigen (PSA), one of the androgen-responsive genes, is a clinically important marker used to monitor diagnosis, progression, and prognosis of patients with prostate cancer. By using Western, Northern blots and EMSA analysis, we found that genistein at low concentration (<10 μM) transcriptionally down-regulated AR, decreased nuclear protein binding to androgen-responsive element (ARE) and, thereby, inhibited the transcription and protein expression of PSA in androgen-sensitive LNCaP cells [85,86] (Fig. 2). However, higher concentrations (10–50 μM) of genistein were needed to significantly inhibit PSA secretion in androgen-insensitive VeCaP cells without alternations in the AR expression and ARE binding activity. These results suggest that genistein may be a powerful agent in the inhibition of PSA. We further transfected a PSA promoter-reporter construct into LNCaP and VeCaP cells followed by treatment with or without genistein (0.5–50 μM) in the presence of media with or without R1881, a synthetic androgen. We found that genistein inhibited PSA synthesis in prostate cancer cells through both androgen-dependent and androgen-independent pathway, suggesting that genistein may act as a chemopreventive and/or therapeutic agent for prostate cancer irrespective of androgen responsiveness. Fritz et al. found that dietary genistein down-regulated expression of AR in the rat prostate at concentrations comparable to those found in humans on a soy diet [87]. Down-regulated AR expression may be responsible for the lower incidence of prostate cancer in populations on a diet containing high levels of phytoestrogens. Another chemopreventive agent EGCG also showed a dose-dependent inhibition of cell growth in both androgen-insensitive DU145 and androgen-sensitive LNCaP cells [80].

Similar to our results about the effects of genistein on AR pathway in prostate cancer cells, Le et al. also reported that DIM inhibited cell proliferation, endogenous PSA transcription, and intracellular and secreted PSA protein expression induced by dihydrotestosterone (DHT) in LNCaP cells [88]. They found that DIM inhibited androgen-induced androgen receptor (AR) translocation into the nucleus. Results of receptor binding assays indicated that DIM was a strong competitive inhibitor of DHT binding to the AR, suggesting that DIM is a strong androgen antagonist in human prostate cancer cells. Gupta et al. also reported that apigenin treatment resulted in a significant decrease in AR protein expression along with a decrease in intracellular and secreted forms of PSA in LNCaP cells [33].

The effects of curcumin on cell growth, activation of signal transduction, and transforming activities in both androgen-dependent and -independent cell lines have been evaluated. Nakamura et al. have found that curcumin down-regulates transactivation and expression of AR and AR-related cofactors (AP-1 and NF-κB), and reduces colony forming ability in soft agar [89]. A number of curcumin analogues was evaluated as potential androgen receptor antagonists against two human prostate cancer cell lines, PC-3 and DU-145, in the presence of androgen receptor (AR) and androgen receptor coactivator, ARA70 [90]. The results showed that some curcumin analogues possessed potent anti-androgenic activities and were superior to hydroxyflutamide, which is the currently available anti-androgen for the treatment of prostate cancer. Structure–activity relationship (SAR) studies demonstrated that some moieties seem to be important factors related to the anti-androgenic activity. These results suggest that these compounds may serve as a new class of anti-androgen agents to control androgen receptor-mediated prostate cancer growth.

7. Effects of chemopreventive agents on ER

Many environmental chemicals have been found to be estrogenic and have been shown to stimulate the growth of ER-positive human breast cancer cells [91,92]. Because it is difficult to avoid human exposure to environmental estrogens, it is important to develop dietary strategies to prevent the stimulated growth of breast tumors by environmental estrogens. Isoflavone has a close similarity in structure to estrogen, and has been known as phytoestrogen. Because of the structural similarity to estrogen, isoflavones have been believed to exert their effects through ER signaling pathway. However, experimental study has found that isoflavones at different concentration may exhibit different effects [93]. Genistein at concentrations ≤ 1 μM may induce breast cancer cell proliferation by estrogenic agonistic properties, while genistein at concentrations ≥ 5 μM may prevent hormone-dependent growth of breast can-

cer cells by potential estrogen-antagonistic activity. Fritz et al. found that dietary genistein down-regulated expression of ER-α and -β in rat at concentrations comparable to those found in humans on a soy diet [87]. Recent studies from Chen et al. [94] showed that genistein at 50 and 100 μM significantly arrested the growth of MCF-7 cells at G2/M phase and down-regulated mRNA expression of ERα, suggesting that the inhibitory action of genistein on human breast cancer cells appears to be partially mediated by the alteration of estrogen receptor-dependent pathways. However, experimental studies also showed that isoflavones exert their inhibitory effects on ER-negative MDA-MB-231 breast cancer cells [95] and hormone-independent cancer cells [78,79,96–99]. These results suggest that isoflavones may exert their effects through ER-dependent (Fig. 2) or independent pathway.

I3C has been known to be a negative regulator of estrogen. When cells were treated with I3C and genistein, a synergistic effect of I3C and genistein was observed on the increase in GADD (growth arrest and DNA damage) expression, the induction of apoptosis, and the decrease in gene expression driven by ERα in MCF-7 breast cancer cells [100]. I3C significantly repressed the transcriptional activity of ERα, the estradiol-activated ERα signaling, and the expression of the estrogen-responsive genes, pS2 and cathepsin-D [101]. These results suggest that anti-tumor activities of I3C are associated not only with its regulation of estrogen activity and metabolism, but also its modulation of ER transcription activity. However, I3C has also been found to inhibit the expression of CDK6 and induces a G1 cell cycle arrest of human breast cancer cells, independent of estrogen-receptor signaling [102].

The effects of curcumin on ER signaling pathway have been investigated in ER-positive human breast cancer line MCF-7 and ER-negative human breast cancer line MDA-MB-231 [103]. The results showed that curcumin inhibited the proliferation of both ER-positive and ER-negative cells. The antiproliferative effect of curcumin was estrogen dependent in ER-positive MCF-7 cells. It has been reported that curcumin inhibited the expression of ER downstream genes including pS2 and TGF-α in ER-positive MCF-7 cells, and this inhibition was dependent on the presence of estrogen [17]. However, curcumin also exerted strong anti-invasive effects in vitro in ER-negative MDA-MB-231 breast cancer cells, and this effect was not estrogen dependent [17]. These results suggest that curcumin may exert its chemopreventive effects through ER-dependent or -independent pathway.

Because both genistein and curcumin showed inhibitory effects on ER-positive and -negative breast cancer cells, the inhibitory effects of a combination of curcumin and genistein were studied in ER-positive human breast cancer cells (MCF-7 and T47D) and ER-negative MDA-MB-231 cells [103]. The results showed that combination of curcumin and genistein significantly inhibited the growth of ER-positive cells. For ER-negative MDA-MB-231 cells, the IC50 for curcumin was 17 μM, which was reduced to 11 μM in the presence of 25 μM genistein. Curcumin and genistein also induced drastic changes in the morphological shape of both ER-positive and -negative cells. These results suggest that combination of natural plant compounds may have stronger preventive and therapeutic effects against the growth of breast cancers.

EGCG has been found to bind to ERα and ERβ, and elicit ER-mediated gene expression in vitro. It has been found that EGCG at higher dose is anti-estrogenic for ERα, however, it is estrogenic for ERα and also for ERβ at lower doses [104]. The in vitro and in vivo studies have demonstrated that polyphenolic catechins (EGCG and ECG) from green tea bind to ERα and ERβ, and inhibited breast cancer cell proliferation and tumor growth, but only EGCG elicited ER-mediated gene expression [105], suggesting that polyphenolic catechins may exert their chemopreventive effects through ER-dependent or independent pathway.

8. Summary and perspectives

The data from in vivo human and animal studies and in vitro experiments clearly indicate that natural chemopreventive agents exert their inhibitory effects on carcinogenesis and tumor progression. These effects have been believed to be mediated through the regulation of cell signaling pathways including NF-κB, Akt, MAPK, p53, AR, and ER pathways. As we discussed earlier, there are cross-talks between these pathways. Natural chemopreventive agents could exert their effects on these pathways separately or sequentially. By modulating cell signaling pathways, chemopreventive agents activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibi-

tion of cancer development or progression. It appears that the effects of these chemopreventive agents are not affected by the endogenesis molecular status such as p53 mutations and ER status of cancer cells. However, the regulation of cell signaling pathways by natural chemopreventive agents is important event in the prevention of cancers. More in depth in vitro and in vivo experiments are needed to fully elucidate the molecular mechanisms of action of chemopreventive agents in future studies.

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SELECTIVE GROWTH REGULATORY AND PRO-APOPTOTIC EFFECTS OF DIM IS MEDIATED BY AKT AND NF-kappaB PATHWAYS IN PROSTATE CANCER CELLS

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1. ABSTRACT

Prostate cancer is the second leading cause of cancer related deaths in men in the United States. I3C and its *in vivo* dimeric product, DIM, have been found to inhibit the growth of prostate cancer cells. However, the molecular mechanism(s) by which DIM elicits its effects on prostate cancer cells has not been fully elucidated. We have previously shown that I3C induces apoptosis and inhibits the activation of NF-kappaB pathway, which could be mediated via Akt signaling pathway. In this study, we investigated whether there is any cross-talk between Akt and NF-kappaB during DIM-induced apoptosis in PC-3 prostate cancer cells. We found that DIM inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells but not in non-tumorigenic CRL2221 human prostate epithelial cells. DIM also inhibited EGFR expression, PI3K kinase activity, and Akt activation, and abrogated the EGF-induced activation of PI3K in prostate cancer cells. NF-kappaB DNA-binding analysis and transfection studies with Akt cDNA constructs revealed that Akt transfection resulted in the induction of NF-kappaB activity and this was inhibited by DIM treatment. DIM treatment also showed significant induction of apoptosis in non-transfected cells compared to Akt and Akt-Myr transfected prostate cancer cells. From these results, we conclude that the inhibition of Akt and NF-kappaB activity and their cross-talk is a novel mechanism by which DIM inhibits cell growth and induces apoptotic processes in prostate cancer cells but not in non-tumorigenic prostate epithelial cells.

2. INTRODUCTION

Prostate cancer is one of the most common cancers in men and the second leading cause of male cancer death in the United States (1). However, Asians have relatively low incidence of prostate cancers. Dietary and epidemiological studies have shown an association between high dietary intake of vegetables and decreased prostate cancer risk (2, 3). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family including broccoli, cabbage, brussels sprouts, and cauliflower appears to be most effective at reducing the risk of cancers. Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family, and it is readily converted to its dimeric product, 3,3'-diinolylmethane (DIM) (4). There are growing evidences showing that I3C and DIM have the potential to inhibit a number of common cancers, especially those that are hormone-related (5-8).

It has been demonstrated that I3C and DIM possess anti-carcinogenic effects in experimental animals and inhibits the growth of human cancer cells *in vitro* (5, 7-10). DIM has been found to induce cell cycle arrest at G1 phase with up-regulation of $p21^{WAF1}$ and down-regulation of CDK6 (6, 11). It has been reported that DIM increases the expression of Bax, decreases the expression of Bcl-2, and induces apoptosis (12). Because of these effects, the interest in I3C and DIM as cancer chemopreventive and/or

therapeutic agents has significantly increased in the past years. We have previously shown that I3C up-regulates $p21^{WAF1}$, Bax, and $p27^{KIP1}$, and down-regulates Bcl_{XL}, EGFR, and Akt kinase activity, leading to the induction of apoptosis in prostate cancer cells (13, 14). We have also reported the gene expression profiles of prostate cancer cells exposed to I3C and DIM, showing that I3C and DIM induce the expression of genes related to the Phase I and Phase II enzymes and regulate the expression of genes involved in the control of cell growth, cell cycle, apoptosis, signal transduction, and oncogenesis (15). However, the precise molecular mechanism by which DIM exerts its effect on the induction of apoptosis and the cell signaling pathways, has not been fully elucidated.

Akt and NF-kappaB pathways are important cell signaling pathways involved in the processes of apoptosis, carcinogenesis, and tumor progression (16-19). NF-kappaB is a cell survival factor and can be activated by many types of stimuli including TNF-α, EGF, UV radiation, etc. There is growing evidence to suggest the role of NF-kappaB in the protection against apoptosis (18, 20). An *in vivo* study showed that mice lacking NF-kappaB p65/RelA died embryonically from extensive apoptosis in the liver (21), suggesting anti-apoptotic role of NF-kappaB. Akt can be activated by various growth factors including EGF through activation of phosphatidylinositol-3 kinase (PI3K) (17). Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate downstream targets (16, 17). Several reports have showed that Akt also regulates the NF-kappaB pathway via phosphorylation and activation of molecules in the NF-kappaB pathway (22-24). Because both Akt and NF-kappaB have been critically involved in the cell survival and apoptotic process, in this study, we investigated whether DIM could inhibit Akt and NF-kappaB activation leading to apoptosis, and whether Akt and NF-kappaB pathways could cross-talk during apoptotic process induced by DIM. Finally we also investigated whether there is any differential effect of DIM between PC-3 prostate cancer cells and non-tumorigenic CRL2221 prostate epithelial cells.

3. MATERIALS AND METHODS

3.1. Cell culture and reagents

PC-3 human prostate cancer cells (ATCC, Manassas, VA) were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. CRL-2221 human non-tumorigenic prostate epithelial cells (ATCC, Manassas, VA) were cultured in keratinocyte-SFM media (Invitrogen, Carlsbad, CA) supplemented with EGF (0.2 microgram/L), bovine pituitary extract (30 milligram/L), and 1% penicillin and streptomycin. DIM (LKT, St. Paul, Minnesota) was dissolved in DMSO to make 60 millimole/L stock solution. Wherever indicated, EGF (Invitrogen, Carlsbad, CA) was added to the media at a final concentration of 100 microgram/L.

3.2. Cell growth inhibition by MTT assay

The PC-3 and CRL-2221 cells were seeded at a density of 1×10^3/well in 96 well culture dishes. After 24 hours, the cells were treated with 15, 30, and 60 micromole/L DIM for one to three days. Control PC-3 cells received 0.1% DMSO for same time points. The cells were then incubated with MTT (0.5 gram/L, Sigma, St. Louis, MO) at 37°C for 4h and with DMSO at room temperature for 1 h. The spectrophotometric absorbance of the samples was measured by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 595 nm. The experiment was repeated three times and t test was performed to verify the significance of cell growth inhibition after treatment.

3.3. Histone/DNA ELISA for detecting apoptosis

Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA, USA) was used to detect apoptosis in PC-3 and CRL-2221 cells with different treatments according to manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from PC-3 and CRL-2221 cells treated with 15, 30, and 60 micromole/L DIM or 0.1% DMSO (vehicle control) for 24, 48, 72 hours, were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC) at 405 nm.

3.4. Western blot analysis

The PC-3 and CRL-2221 cells were plated on culture dishes and allowed to attach for 24 hours followed by the treatment with 15, 30, or 60 micromole/L DIM for 48 hours. Control cells were incubated in the medium with 0.1% DMSO using same time points. After incubation, the cells were lysed in 62.5 millimole/L Tris-HCl and 2% SDS. Protein concentration was then measured using BCA protein assay (PIERCE, Rockford, IL). Cell extracts were subjected to 10% SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with anti-Akt (Santa Cruz Biotech, Santa Cruz, CA), anti-phospho-Akt Ser473 (Cell signaling, Beverly, MA), anti-EGFR (Santa Cruz Biotech, Santa Cruz, CA), anti-Bcl_{XL} (Santa Cruz Biotech, Santa Cruz, CA), and anti-β-actin (Sigma, St. Louis, MO) antibodies, washed with TTBS and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (PIERCE, Rockford, IL).

3.5. Reporter gene constructs and transfection

pLNCX-Akt (normal Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control vector) were generously provided by Dr. Sellers (Dana-Farber Cancer Institute, Boston, MA). NF-kappaB-LUC (Stratagene, La Jolla, CA) contains six repeated copies of the NF-kappaB DNA-binding site and a luciferase reporter gene. CMV-beta-gal reporter construct transfection was used for normalization of transfection efficiency. The pLNCX-Akt,

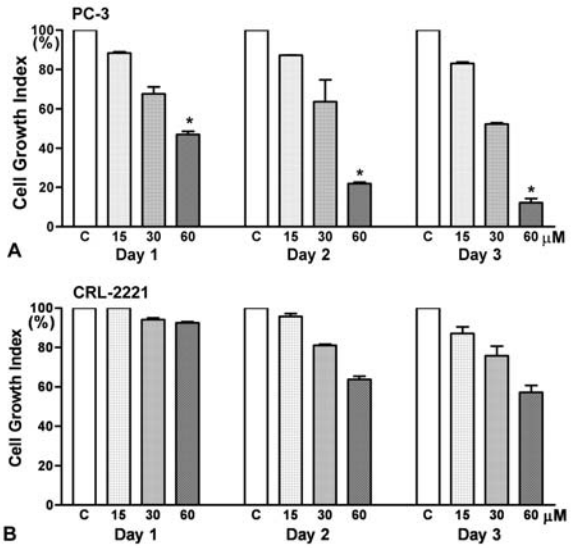

Figure 1. Effects of DIM on the growth of PC-3 (A) and CRL-2221 cells (B) tested by MTT assay. (*: $p<0.05$; n=3).

pLNCX-Myr-Akt, pLNCX-Akt-K179M, or pLNCX was transiently co-transfected with NF-kappaB-LUC and CMV-beta-gal into PC-3 cells when they were at ~70% confluent using the LipofectAMINE (Invitrogen, Carlsbad, CA). After incubation for 5 hours, the transfected cells were washed and incubated with RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum overnight followed by treatment with 60 micromole/L DIM for 48 hours. Subsequently, the luciferase activities in the samples were measured by using Steady-Glo™ Luciferase assay system (Promega, Madison, WI) and ULTRA Multifunctional Microplate Reader (TECAN, Durham, NC). To detect the NF-kappaB activity in transfected PC-3 cells, the samples were subjected to NF-kappaB DNA-binding activity measurement using EMSA method as described below. Cell Apoptosis ELISA Detection Kit was also used to detect apoptosis in transfected and parental PC-3 cells.

3.6. NF-kappaB DNA-binding activity measurement

PC-3 and CRL-2221 cells were treated with 15, 30, and 60 micromole/L DIM for 72 hours. Following treatment, the nuclear proteins from cells were extracted. Ten microgram of nuclear proteins was subjected to electrophoretic mobility shift assay (EMSA) as described previously (25). Competition assay using unlabeled specific competitor (NF-kappaB oligo) was conducted to confirm the specificity of NF-kappaB DNA-binding activity.

3.7. Immunoprecipitation and PI3K kinase assay

The PI3K kinase activity of PC-3 cells treated with 60 micromole/L DIM for 24 and 48 hours, 60 micromole/L DIM for 24 and 48 hours followed by EGF treatment for 20 minutes, EGF only, or 0.1% DMSO was measured by using PI3K kinase assay kit (Echelon, Salt Lake, UT) according to manufacturer's protocol. Briefly, the cells after treatments were lysed in ice-cold cell lysis buffer (137 millimole/L NaCl, 20 millimole/L Tris-HCl, 1 millimole/L $CaCl_2$, 1 millimole/L $MgCl_2$, 0.1 millimole/L sodium orthovanadate, 1% NP-40, and 1 millimole/L PMSF) on ice for 20 minutes. After centrifugation, the protein concentration of supernatant was measured by BCA protein assay (PIERCE, Rockford, IL). 600 micrograms of proteins from each sample were used for immunoprecipitation with PI3K antibody (Upstate, Charlottesville, VA) overnight and protein G-agarose for one hour at 4°C. Then, the samples were collected by centrifugation, washed with kinase buffer, and subjected to PI3K kinase assay in kinase buffer (20 millimole/L Tris pH 7.4, 4 millimole/L $MgCl_2$, 10 millimole/L NaCl), 25 micromole/L ATP, and 2.4 micrograms of $PI(4,5)P_2$ as kinase substrate. PI(3,4,5)P3 was measured by competitive ELISA.

3.8. Signal quantification and statistical analysis

The EMSA gel was scanned, and the signals in the gel were quantified and analyzed with Odyssey software (LI-COR, Lincoln, NE). Signal in the Western blots was also scanned and quantified with Molecular Analyst software (Bio-Rad, Hercules, CA). Comparisons were made between control and treatments. Statistical analysis was performed using t test between treated and untreated samples. P values less than 0.05 were used to indicate statistical significance.

4. RESULTS

4.1. DIM selectively inhibits growth of prostate cancer cells

PC-3 prostate cancer cells and CRL-2221 non-tumorigenic human prostate epithelial cells were treated with 0-60 micromole/L DIM over 3 days and the cell viability was determined by MTT assay. The treatment of PC-3 prostate cancer cells with DIM resulted in a dose and time-dependent inhibition of cell proliferation (Figure 1A). However, only 37.8% growth inhibition was observed in the CRL-2221 non-tumorigenic prostate epithelial cells treated with 60 micromole/L DIM for 3 days compared to 87.8% in PC-3 cells (Figure 1B), suggesting the selective growth inhibition of prostate cancer cells by DIM. Inhibition of cell proliferation observed by MTT could be partly due to the induction of apoptosis in prostate cancer cells. We, therefore, investigated whether DIM could selectively induce apoptosis in PC-3 prostate cancer cells.

4.2. DIM selectively induces apoptosis in prostate cancer cells

By ELISA analysis of cytoplamic histone/DNA fragments, we observed an induction of apoptosis in prostate cancer cells treated with 15-60 micromole/L DIM (Figure 2A). The induction of apoptosis was time- and dose-dependent, and was directly correlated with the inhibition of cell growth, suggesting that DIM treatment may result in the inhibition of cell proliferation through apoptotic cell death. More importantly, non-tumorigenic CRL-2221 prostate epithelial cells were much less responsive to DIM treatment than PC-3 cells (Figure 2B), suggesting that DIM selectively induced apoptosis in prostate cancer cells.

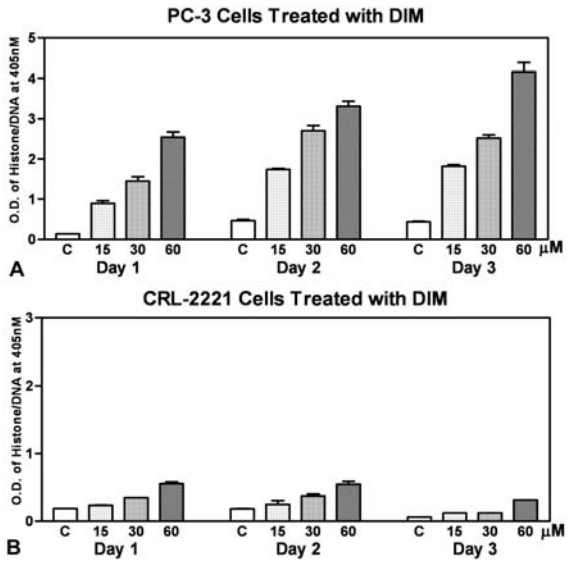

Figure 2. Induction of apoptosis in PC-3 (A) and CRL-2221 (B) cells tested by ELISA. (n=2).

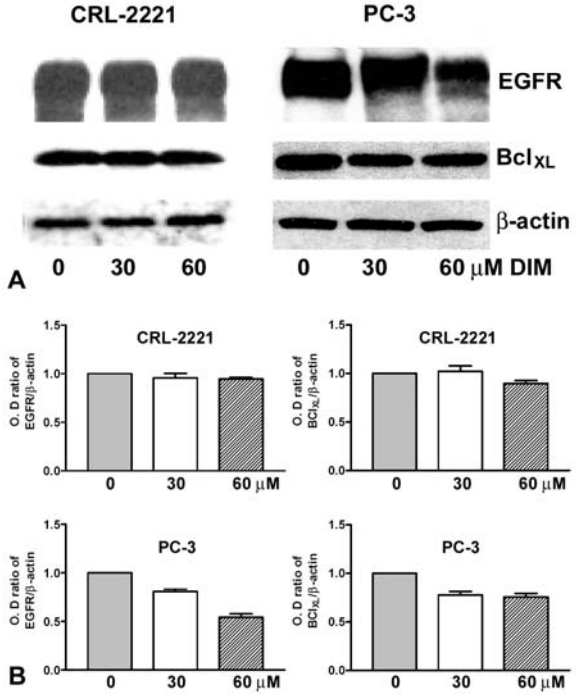

Figure 3. Western blot (A) and densitometric analysis (B) of Bcl_{XL} and EGFR in PC-3 and CRL-2221 cells treated with DIM.

By Western Blot analysis, we also found that DIM inhibited the expression of Bcl_{XL}, an anti-apoptotic protein in PC-3 cells (Figure 3). However, there was no significant effect on Bcl_{XL} in DIM-treated CRL-2221 cells, and these results were correlated with minimal apoptosis in DIM-treated CRL-2221 cells. Next, we investigated whether PI3K/Akt and NF-kappaB signaling pathways are involved in the apoptotic processes induced by DIM in prostate cancer cells.

4.3. DIM inhibits PI3K and Akt activation and induces apoptoisis through Akt pathway

Since Akt signaling pathway is an important signal transduction pathway that plays a critical role in cell survival and apoptotic processes, we investigated the status of Akt in PC-3 and CRL-2221 cells treated with 0-60 micromole/L DIM by Western Blot analysis. We did not find any alterations in the protein expression of un-phosphorylated Akt in DIM-treated PC-3 and CRL-2221 cells (Figure 4). However, a significant decrease in the phosphorylated Akt protein at Ser473 was observed in DIM treated PC-3 cells compared to control cells, suggesting inactivation of Akt kinase after DIM treatment (Figure 4). Treatment of DIM showed dose dependent inhibition of Akt phosphorylation in PC-3 cells, consistent with the induction of apoptosis by DIM. However, the phosphorylated Akt Ser473 protein was undetectable in CRL-2221 cells treated or untreated with DIM, suggesting that the inactivation of Akt by DIM is specific in prostate cancer cells compared to non-tumorigenic prostate epithelial cells.

Since Akt is activated through the activation of PI3K, we investigated the PI3K kinase activity in the PC-3 cells treated with DIM or pre-treated with DIM followed by EGF stimulation. We found that DIM treatment inhibited the activity of PI3K (Figure 5), suggesting that DIM could inactivate Akt through the inhibition of PI3K activity. We also found that EGF treatment alone activated PI3K kinase activity as expected, and that DIM pretreatment abrogated the activation of PI3K stimulated by EGF (Figure 5). Because the activation of Akt and PI3K could be mediated through EGFR pathways, we measured the expression of EGFR by Western Blot analysis. We found that DIM inhibited EGFR expression in a dose-dependent manner in PC-3 cells only and showed no inhibition of EGFR in CRL-2221 cells (Figure 3), corresponding with the selective effect of DIM on Akt activation in PC-3 cells.

Furthermore, we transfected Akt cDNA into PC-3 cells and measured the degree of apoptosis in transfected cells with and without DIM treatment. We found that DIM not only induced apoptosis in PC-3 parental cells but also in Akt transfected PC-3 cells although to a lesser extent as expected (Figure 6). More importantly, we found that transfection of constitutively activated Akt (pLNCX-Myr-Akt) and wild-type Akt (pLNCX-Akt) inhibited apoptosis induced by DIM compared to mutant Akt and empty vector transfectants, suggesting that the induction of apoptosis by DIM is partly mediated by active Akt and that the overexpression of Akt leads to resistance to DIM-induced apoptosis.

4.4. DIM selectively inhibits NF-kappaB activation in PC-3 cells

Nuclear extracts from control and DIM-treated PC-3 cells were subjected to NF-kappaB DNA-binding activity as measured by EMSA. Autoradiography revealed that 30-60 micromole/L DIM significantly inhibited NF-kappaB DNA-binding activity in PC-3 cells compared to untreated control (Figure 7). However, no such effect was observed in DIM-treated CRL-2221 cells, suggesting the

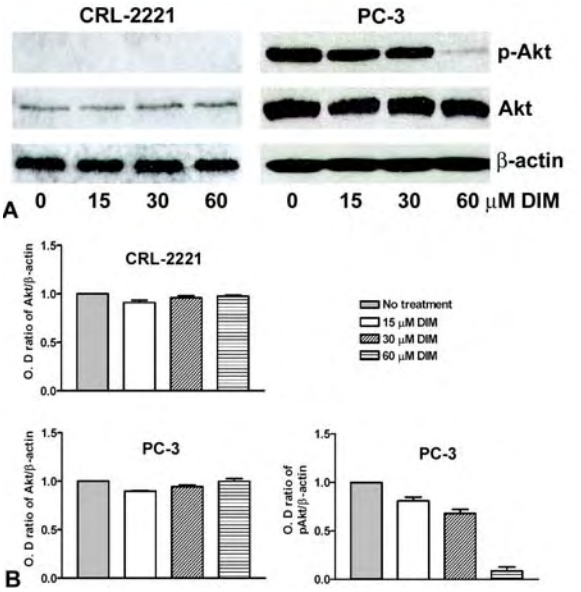

Figure 4. Western blot (A) and densitometric analysis (B) of total Akt and p-Akt in PC-3 and CRL-2221 cells treated with DIM.

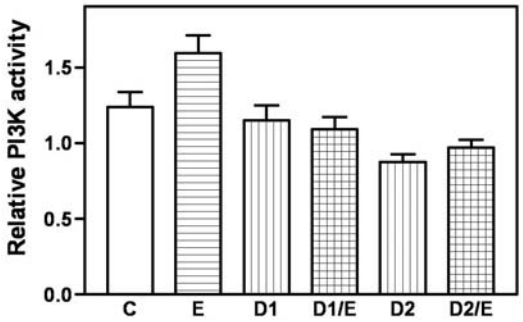

Figure 5. Relative PI3K activity in PC-3 cells treated with DIM. (C: control; E: treated with EGF; D1: treated with 60 micromole/L DIM for 24 hours; D1/E: treated with 60 micromole/L DIM for 24 hours followed by EGF treatment; D2: treated with 60 micromole/L DIM for 48 hours; D2/E: treated with 60 micromole/L DIM for 48 hours followed by EGF treatment).

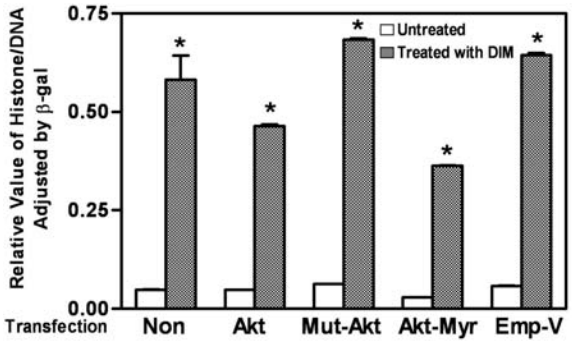

Figure 6. Induction of apoptosis in transfected and DIM treated PC-3 cells tested by ELISA (Non: no transfection; Akt: transfected with pLNCX-Akt; Mut-Akt: transfected with pLNCX-Akt-K179M; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

selective inhibitory effect of DIM on NF-kappaB DNA-binding activity in prostate cancer cells. In order to further explore the inhibitory effects of DIM on Akt and NF-kappaB pathways, we conducted transfection experiments as described under Materials and Methods. Luciferase assay showed a significant increase in luciferase activity in PC-3 cells co-transfected with pLNCX-Myr-Akt and NF-kappaB-Luc, and also in PC-3 cells co-transfected with pLNCX-Akt and NF-kappaB-Luc (Fig 8). Moreover, DIM significantly abrogated the induction of luciferase activity caused by pLNCX-Myr-Akt and pLNCX-Akt transfections (Figure 8).

To confirm these results, we also examined the NF-kappaB DNA-binding activity in PC-3 cells transfected with pLNCX-Myr-Akt or pLNCX. We observed an increase in NF-kappaB DNA-binding activity in PC-3 cells transfected with pLNCX-Myr-Akt (Figure 9). We also found that DIM abrogated the activation of NF-kappaB DNA-binding activity caused by pLNCX-Myr-Akt transfection (Figure 9). Collectively, these results provide evidence for a potential cross-talk between Akt and NF-kappaB pathways during DIM induced cell growth inhibition and apoptosis in prostate cancer cells.

5. DISCUSSION

DIM, the major *in vivo* product of dietary I3C, has been shown to inhibit cell growth and induce apoptosis in breast, cervical and prostate cancer cells (5-7, 11), suggesting its chemopreventive and/or therapeutic effects on cancer cells. However, the precise molecular mechanisms by which DIM inhibits cell growth and induces apoptosis have not been fully elucidated. Additionally, the effect of DIM on non-tumorigenic epithelial cells remains unknown. Here, we demonstrated that DIM significantly and selectively inhibited cell growth and induced apoptosis in PC-3 prostate cancer cells, while CRL-2221 non-tumorigenic cells showed much less response to DIM. These results provide evidence for selective effects of DIM on cell growth and apoptosis in cancer cells. To discover the molecular mechanisms responsible for the induction of apoptosis by DIM, we investigated the effects of DIM on Akt and NF-kappaB pathways, which have been known to play important roles in cell survival and apoptotic cell death processes.

It has been known that Akt signaling pathway can be activated by various growth and survival factors such as EGF, PDGF, insulin, etc, through activation of PI3K (16, 17). PI3K activation leads to the production of phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3), which interacts with Akt PH domain. This interaction subsequently causes conformational changes in Akt, resulting in the exposure of two 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. Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets including Bad, Forkhead transcription factors, and caspase-9, all of which are involved in apoptotic pathway (26, 27). In the apoptotic

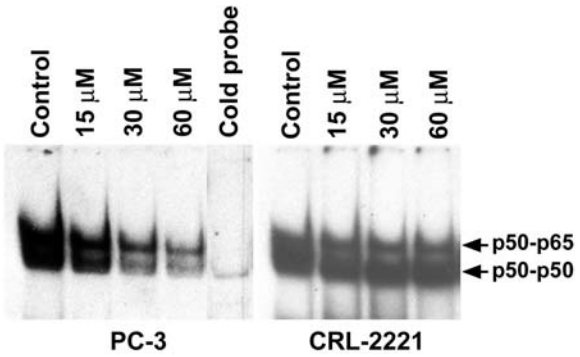

Figure 7. NF-kappaB DNA-binding activity in DIM treated PC-3 and CRL-2221cells tested by EMSA. (Cold probe: Unlabeled NF-kappaB oligonucleotide was used as specific competitor in DNA-binding reaction).

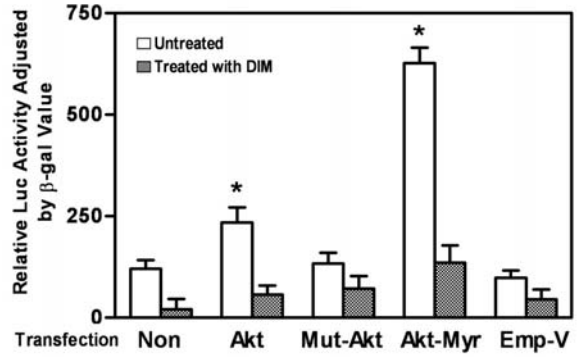

Figure 8. Luciferase activity in transfected PC-3 cells with or without DIM treatments (Non: no transfection; Akt: transfected with pLNCX-Akt; Mut-Akt: transfected with pLNCX-Akt-K179M; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

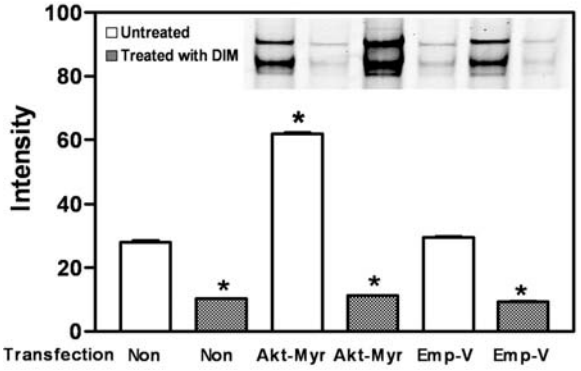

Figure 9. EMSA and densitometric analysis of NF-kappaB DNA-binding activity in transfected PC-3 cells with or without DIM treatments (Non: no transfection; Akt-Myr: transfected with pLNCX-Myr-Akt; Emp-V: transfected with empty vector, pLNCX; *: $p<0.05$; n=2).

process, dephosphorylated Bad (activated form of Bad) translocates to mitochondria, where it heterodimerizes via its BH3 domain with anti-apoptotic BCL family members such as Bcl-2 and Bcl_{XL}, promoting the onset of apoptosis (28-31). In this study, we found that DIM treatment caused the down-regulation of EGFR, suggesting that DIM could inhibit the activation of PI3K through decrease in growth factor binding. By PI3K kinase assay, we found that DIM inactivated PI3K and abrogated the activation of PI3K caused by EGF, suggesting that DIM could inhibit Akt activity by inactivation of PI3K. Subsequently, we found no changes in the expression of un-phosphorylated Akt in DIM treated PC-3 cells. However, DIM decreased the level of phosphorylated Akt, which is the activated form of Akt. This could subsequently increase the activated Bad binding to Bcl-2 and Bcl_{XL}. We found that DIM inhibited the expression of Bcl_{XL}, suggesting an increase in the ratio of Bad/Bcl_{XL}, which could promote cancer cell to apoptotic cell death. Indeed, we observed significant induction of apoptosis in DIM treated PC-3 prostate cancer cells, and lesser apoptosis in Akt transfected PC-3 cells compared to parental PC-3 cells. These results suggest that the inhibition of PI3K/Akt signaling pathway by DIM is one of the mechanisms by which DIM induces apoptosis in prostate cancer cells.

It has been well known that NF-kappaB plays an important role in the apoptotic process (18, 20). Thus, DIM may induce apoptosis by modulating multiple components in the Akt and NF-kappaB pathways. In this study, we found that DIM selectively inhibited NF-kappaB DNA-binding activity in PC-3 prostate cancer cells. The inactivation of NF-kappaB DNA-binding activity may be another mechanism by which DIM induces apoptosis in PC-3 cells. It has been reported that the activity of NF-kappaB may be regulated by a variety of factors including Akt (22, 24). Akt has been shown to enhance the degradation of the IkappaB and induce NF-kappaB activation (32). The ability of Akt to regulate NF-kappaB activity may be through direct interaction with the IKK, supported by the observation that Akt is associated with the IKK complex *in vivo* (33). It has been demonstrated that Akt can phosphorylate and activate IKK at a critical regulatory site, Thr23, and subsequently activate NF-kappaB (24). In this study, we transfected Akt to PC-3 prostate cancer cells and tested the effect of Akt on NF-kappaB DNA-binding activity by EMSA and luciferase assay. Our results showed that Akt regulated NF-kappaB activation, and this observation is in accordance with our published data (25). Importantly, we found that DIM abrogated the NF-kappaB activation stimulated by Akt transfection, suggesting that the inhibition of NF-kappaB activity by DIM is partly mediated through Akt signaling pathway. Hence, the inactivation of DNA-binding activity of NF-kappaB by DIM appears to be responsible for DIM-induced apoptosis in PC-3 prostate cancer cells.

NF-kappaB has been described as a major culprit in cancer (34), and Akt has been known as a key molecule in cell survival (16, 17). Because of their importance in the control of cell survival and apoptotic cell death, both Akt and NF-kappaB have been believed to be very attractive therapeutic targets for cancer therapy (33, 35-37). Therefore, our results indicate that the inhibition of Akt and NF-kappaB activity could be easily achievable by DIM treatment, which inhibits cell growth and induces apoptosis in PC-3 prostate cancer cells, suggesting that DIM may be a useful agent for the prevention and/or treatment of

prostate cancer. More importantly, we found that DIM had no significant effects on cell growth, apoptosis, Akt and NF-kappaB activity in non-tumorigenic prostate epithelial cells, suggesting cancer cell specific effects of DIM. Similar cancer cell specific effects of I3C were also reported previously by our laboratory in breast epithelial cells (38). The fact that DIM selectively inhibits cell growth, Akt and NF-kappaB activation, and induces apoptosis in PC-3 prostate cancer cells, makes it a potent chemopreventive and/or therapeutic agent against prostate cancer. Our results warrant further animal and human investigations in order to fully appreciate the value of DIM in human health.

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Appendix 7

[Proc Amer Assoc Cancer Res, Volume 46, 2005]

Clinical Research 2: Novel Therapeutics and Biomarkers of Response

Abstract #466

Down regulation of androgen receptor (AR) and its target gene (PSA) expressions by B-DIM leads to sensitization of prostate cancer cells to Taxotere-induced apoptosis.

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Despite initial response to androgen deprivation, patients with prostate cancer usually develop hormone refractory metastasis and eventually succumb to the disease. Therefore, new therapeutic strategies are urgently needed to overcome drug resistance for the treatment of prostate cancer. Epidemiological studies have shown that the consumption of cruciferous vegetables is associated with reduced cancer risk. 3,3'-diindolymethane (DIM) is an acid condensation product of I3C, which is present in almost all members of the cruciferous vegetable family. DIM has been shown to down-regulate AR, suggesting the growth inhibitory effects of DIM on hormone related cancers. A formulated DIM (B-DIM, BioResponse) has been shown to exhibit approximately 50% higher bioavailability. However, no molecular studies have been reported to date to elucidate the effect of B-DIM on prostate cancer cells. To investigate the effects of B-DIM on prostate cancer cells, we treated hormone sensitive LNCaP cells and hormone insensitive PC-3 cells with 0 to 60 µM of B-DIM for one to three days. MTT, ELISA, and Western blot analysis were conducted to test cell growth inhibition, apoptosis, and the expression of AR and prostate specific antigen (PSA). Microarray analysis was also conducted to assess global alternations in gene expression profile. We found that B-DIM inhibited the growth of prostate cancer cells and induced apoptosis in a dose and time dependent manner. Microarray gene expression analysis showed that B-DIM regulated the expression of genes which are critical for the control of cell growth and apoptosis. Western blot analysis showed that B-DIM significantly inhibited AR and PSA protein expression in LNCaP prostate cancer cells. It has been recently reported that increases in androgen receptor mRNA and proteins are both necessary and sufficient to convert prostate cancer from a hormone-sensitive to a hormone refractory stage, suggesting that over-expression of AR is an important factor for acquired resistance of prostate cancer to hormone ablation therapy. Since our results

showed decreased AR and PSA expression with concomitant inhibition of cell growth and induction of apoptosis by B-DIM, we suggest that the resistance acquired by prostate cancer cells to androgen ablation therapy could be reversed by B-DIM treatment. Moreover, we tested our hypothesis whether B-DIM could sensitize hormone insensitive PC-3 prostate cancer cells to Taxotere. We found that Taxotere (1 nM) in combination with 50 μM B-DIM exerted significantly more growth inhibitory effects compared to Taxotere or B-DIM treatment alone. In conclusion, our results provide a solid foundation for devising novel therapeutic strategies for the treatment of hormone or chemo-resistant metastatic prostate cancer by including B-DIM, a non-toxic agent, with other therapeutic agents.

Review

Using Chemopreventive Agents to Enhance the Efficacy of Cancer Therapy

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Abstract

Emerging evidence suggests that cancer preventative agents might be combined with chemotherapy or radiotherapy for the more effective treatment of cancer. Recent studies suggest that genistein and other dietary compounds that prevent cancer may enhance the efficacy of cancer therapeutics by modifying the activity of key cell proliferation and survival pathways, such as those controlled by Akt, nuclear factor-κB, and cyclooxygenase-2. In this article, we summarize the findings of recent investigations of chemopreventive agents in combination with cancer treatment regimens. (Cancer Res 2006; 66(7): 3347-50)

Background

Conventional cancer therapies, including surgery, chemotherapy, and radiotherapy, as single modalities have a limited but important role in the overall treatment of most solid tumors. Thus, the strategies of cancer treatment using combined therapies or combined agents with distinct molecular mechanisms are considered more promising for higher efficacy, resulting in better survival. In recent years, more dietary compounds [i.e., genistein, 3,3′-diindolylmethane, indole-3-carbinol (I3C), curcumin, (−)-epigallocatechin-3-gallate (EGCG), resveratrol, etc.] have been recognized as cancer chemopreventive agents because of their anticarcinogenic activity (1). Moreover, these compounds also exert the antitumor activities through regulation of different cell signaling pathways. Therefore, common cancer therapies combined with these dietary compounds may exert enhanced antitumor activity through synergic action or compensation of inverse properties. The combination treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used. In this short article, we review current knowledge of the effects and the molecular mechanisms of the combination treatments published thus far, to give a brief view on the new and emerging field for optimal treatment of cancer patients with better survival.

Antitumor Activity of Common Cancer Therapies Are Potentiated by Chemopreventive Agents

Recently, there has been a growing interest in investigating the effects of genistein and other chemopreventive agents on the inhibition of cancer cell growth in combination with chemotherapeutics or other common therapies. The number of publications regarding potentiated antitumor effects of cancer therapies by chemopreventive agents has dramatically increased in 2005, suggesting that novel combination treatments with common cancer therapies and chemopreventive agents are beginning to receive much attention in cancer research.

Potentiation of chemotherapeutic effects. The *in vitro* and *in vivo* studies from our laboratory and others have shown that the antitumor effects of chemotherapeutic agents could be enhanced by combination treatment with chemopreventive agents. We have reported that genistein *in vitro* potentiated growth inhibition and apoptotic cell death caused by cisplatin, docetaxel, doxorubicin, and gemcitabine in prostate, breast, pancreas, and lung cancers (2–4). We found that pretreatment of cancer cells with 15 to 30 μmol/L genistein before the treatment with lower doses of chemotherapeutic agents caused a significantly greater degree of growth inhibition and apoptotic cell death, suggesting that increased antitumor activities of chemotherapeutic agents with lower toxicity to normal cells could be achieved by introducing genistein into the chemotherapeutic strategy. To investigate whether these phenomena that we observed *in vitro* could also exit *in vivo*, we conducted animal studies. We found that dietary genistein could potentiate the antitumor activities of gemcitabine and docetaxel in a tumor model, resulting in more tumor cell killing and apoptotic cell death (2, 3). By *in vitro* and *in vivo* studies, we also found that genistein could sensitize diffuse large cell lymphoma to cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP) chemotherapy (5). These results suggest that genistein enhances antitumor activities of chemotherapeutic agents both *in vitro* and *in vivo* in multiple tumors.

Other investigators have reported similar observations showing that the antitumor effects of chemotherapeutics could be enhanced by genistein. Hwang et al. recently reported that the combination of genistein and 5-fluorouracil (5-FU) synergistically induced apoptosis in chemoresistant HT-29 colon cancer cells (6). Genistein was also shown to enhance necrotic-like cell death in HER-2 overexpressing breast cancer cells treated with Adriamycin (7). Tanos et al. found that 1 to 10 μg/mL genistein inhibited the growth of dysplastic and malignant epithelial breast cancer cells *in vitro*, and that the addition of tamoxifen has a synergistic/additive inhibitory effect on breast cancer growth (8). These effects were not modulated by estrogen receptor. In addition, genistein and its isoflavone analogues have been found to decrease the side effects of tamoxifen through P450-mediated pathways (9). Thesis results support our findings and suggest the beneficial effects of genistein on cancer chemotherapy.

In addition to genistein, other dietary chemopreventive agents, including curcumin, EGCG, resveratrol, I3C, proanthocyanidin, and vitamin D, have been shown to enhance the antitumor activities of chemotherapeutic agents. A recent report by Lev-Ari et al. showed that curcumin and celecoxib synergistically inhibited the growth of colorectal cancer cells (10). Curcumin also enhanced the antitumor activities of cisplatin, doxorubicin, and Taxol in HA22T/VGH hepatic cancer cells, HeLa cells, or CAOV3 and SKOV3 ovarian cancer cells (11–13). In addition, the combined curcumin and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)

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treatment increased the number of hypodiploid cells and induced DNA fragmentation in LNCaP cells, suggesting a potential use of curcumin to sensitize prostate cancer cells for TRAIL-mediated immunotherapy (14). It has been reported that EGCG and tamoxifen synergistically induced apoptosis and growth inhibition in MDA-MB-231 human breast cancer cells (15). EGCG could also chemosensitize resistant tumor cells to doxorubicin through an increase in the accumulation of doxorubicin in the tumors of human carcinoma xenograft model (16). It has been found that resveratrol from grapes could sensitize non-Hodgkin's lymphoma and multiple myeloma cells to paclitaxel-mediated apoptosis (17). Proanthocyanidin, another compound from grapes, has been reported to enhance doxorubicin-induced antitumor effect and reverse drug resistance in doxorubicin-resistant K562/DOX cells, breast cancer cells, and mouse tumor xenograft models (18, 19). We and others have found that combinations of I3C and cisplatin or tamoxifen cooperate to inhibit the growth of PC-3 prostate and MCF-7 breast cancer cells more effectively than either agent alone (20, 21). In addition, analogues of vitamin D were also shown to potentiate the antiproliferative effect of doxorubicin, cisplatin, and genistein *in vitro* (22). These results clearly show that dietary cancer preventive agents can potentiate antitumor activities of common chemotherapeutics.

Potentiation of radiotherapy. We have investigated the effect of the combination of genistein and radiation on PC-3 prostate cancer cells. We found that the combination of genistein and radiation showed enhanced inhibitory effects on DNA synthesis, cell growth, and colony formation *in vitro* (23). Furthermore, we found that genistein combined with radiation led to a greater control of the growth of the primary tumor and metastasis to lymph nodes than genistein or radiation alone, suggesting that genistein enhanced the radiosensitivity of PC-3 prostate cancer cells (24). A similar report by other investigators showed that genistein enhanced the radiosensitivity of cervical cancer cells through increased apoptosis, prolonged cell cycle arrest, and impaired damage repair (25). Genistein was also shown to enhance radiosensitivity in human esophageal cancer cells *in vitro* (26), suggesting that the enhancement of radiosensitivity by genistein is not cell type dependent. Apart from genistein, another important chemopreventive agent, curcumin, at a low concentration in combination with radiation showed significant enhancement to radiation-induced clonogenic inhibition and apoptosis in PC-3 prostate cancer cells (27). These reports show that the radiotherapy combined with chemopreventive agents can cause more growth inhibition and apoptotic cell death of various cancers compared with monotreatment.

Molecular Mechanisms of Combination Treatment

The molecular mechanisms by which chemopreventive agents potentiate the antitumor effects of cancer therapies have not been fully elucidated. It is known that chemotherapy and radiotherapy can induce drug resistance in cancer cells, resulting in treatment failure. The major culprits involved in the development of drug resistance are multidrug resistance gene, nuclear factor-κB (NF-κB), and Akt. We and other investigators have found that the enhanced antitumor effects by chemopreventive agents could be, in part, through the regulation of NF-κB, Akt, and cyclooxygenase-2 (COX-2) pathways, which play important roles in cell survival (Fig. 1). Chemopreventive agents could also sensitize cancer cells to apoptosis by regulating several important molecules (i.e., Bcl-2, Bcl-X_L, survivin, caspases, $p21^{WAF1}$, etc.) in the apoptotic pathway (Fig. 1).

Regulation of the Akt pathway. Akt pathway is an important cell signaling pathway involved in drug resistance. It has been found that genistein enhanced necrotic-like cell death with the significant inhibition of Akt activity in breast cancer cells treated with genistein and Adriamycin, suggesting that the enhanced growth inhibition of combination is through the inactivation of the Akt pathway (7). Reports from our laboratory and others also showed that activated Akt was inhibited by genistein combined with gemcitabine or radiation in pancreatic, cervical, and esophageal cancer cells, suggesting that enhancement of chemotherapeutic or radiation effects by genistein may be partially mediated by the Akt pathway (3, 25, 26). Bava et al. recently reported that curcumin down-regulated Taxol-induced phosphorylation of Akt, which interacts with NF-κB, suggesting that enhanced antitumor activity by curcumin is through the Akt and NF-κB pathways (12).

Regulation of NF-κB pathway. It has been known that many chemotherapeutic agents induce activity of NF-κB, which causes drug resistance in cancer cells (28). By *in vitro* and *in vivo* studies, we found that NF-κB activity was significantly increased by cisplatin, docetaxel, gemcitabine, and radiation treatment, and that the NF-κB inducing activity of these agents was completely abrogated by genistein pretreatment in prostate, breast, lung, and pancreatic cancer cells, suggesting that genistein pretreatment inactivates NF-κB and may contribute to increased growth inhibition and apoptosis induced by these agents (2–4, 23). We also found that genistein potentiated the antitumor activity of CHOP by inhibition of NF-κB in lymphoma cells (5). Similarly, curcumin has been found to inhibit the activity of NF-κB and sensitize cancer cells to cisplatin or Taxol-induced apoptosis (12, 29).

Regulation of apoptosis pathways. It has been reported that curcumin combined with cisplatin decreased the expression of several apoptosis-related genes, including *c-myc*, *Bcl-X_L*, *c-IAP-2*, *NAIP*, and *XIAP* (11). The combination of curcumin and TRAIL also induced cleavage of procaspase-3, procaspase-8, and procaspase-9; truncation of Bid; and release of cytochrome *c* from the mitochondria in prostate cancer cells, indicating that the apoptotic pathway is triggered in prostate cancer cells treated with combination of curcumin and TRAIL (14). We and others also found that genistein combined with docetaxel or gemcitabine significantly inhibited Bcl-2, Bcl-X_L, and survivin and induced $p21^{WAF1}$, suggesting that combination treatment regulates the important molecules in the apoptotic pathway (2, 3).

Regulation of other pathways. It has been found that the combination of 5-FU and genistein enhanced therapeutic effects in colon cancers through the COX-2 pathway (6). A recent report showed that curcumin or EGCG could down-regulate COX-2 expression without any change of COX-1 expression at both the mRNA and protein levels in colorectal or prostate cancer cells, suggesting that a combination of curcumin or EGCG with chemotherapeutic agents could be an improved strategy for the treatment of colorectal or prostate cancer (10, 30). Indeed, the synergistic growth inhibitory effect of curcumin and celecoxib was found in colorectal cancer cells through inhibition of the COX-2 pathway (10). Apart from the COX-2 pathway, the molecules in cell cycle regulation may also be involved in mechanisms of combination treatment. It has been reported that combination of I3C and tamoxifen caused a more pronounced decrease in cyclin-dependent kinase 2 (CDK2)–specific enzymatic activity, CDK6 expression, and

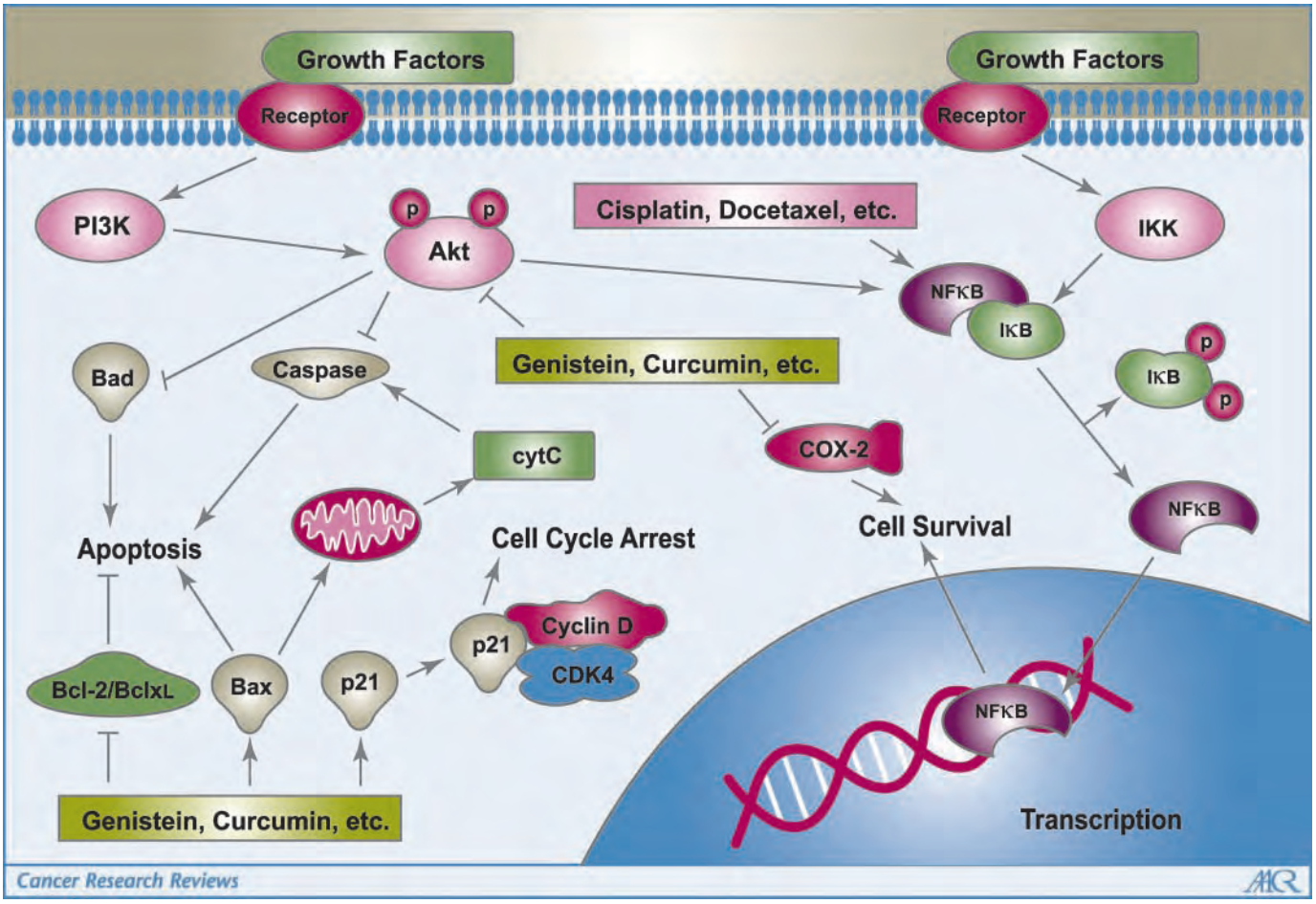

Figure 1. Chemopreventative agents enhance antitumor effects of chemotherapies and radiotherapies through the regulation of Akt, NF-κB, COX-2, and apoptosis pathways. *cytC,* cytochrome *c*.

the level of phosphorylated retinoblastoma protein (20). The enhanced effects of chemotherapy by chemopreventive agents may also be related to immunopotentiating activities through reduction of interleukin-6 (IL-6; ref. 13) and enhancements of lymphocyte proliferation, natural killer cell cytotoxicity, $CD4^+/CD8^+$ ratio, IL-2, and IFN-γ productions (18). In addition, genistein and its isoflavone analogues showed the potential to decrease side effects of tamoxifen through metabolic interactions that inhibit the formation of α-hydroxytamoxifen via inhibition of CYP1A2 (9), suggesting the beneficial effects of genistein in combination with tamoxifen.

Conclusion and Perspective

The *in vitro* and *in vivo* studies reviewed above all suggest that dietary chemopreventive agents may serve as potent agents for enhancing the therapeutic effects of chemotherapy, radiotherapy, or other standard therapeutics for the treatment of human cancers. However, further in-depth mechanistic studies, *in vivo* animal experiments, and clinical trials are needed to bring this concept into practice to fully appreciate the value of chemopreventive agents in combination therapy of human cancers.

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Research Article

Down-regulation of Androgen Receptor by 3,3′-Diindolylmethane Contributes to Inhibition of Cell Proliferation and Induction of Apoptosis in Both Hormone-Sensitive LNCaP and Insensitive C4-2B Prostate Cancer Cells

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Abstract

Despite the initial efficacy of androgen deprivation therapy, most patients with advanced prostate cancer eventually progress to hormone-refractory prostate cancer, for which there is no curative therapy. Previous studies from our laboratory and others have shown the antiproliferative and proapoptotic effects of 3,3′-diindolylmethane (DIM) in prostate cancer cells. However, the molecular mechanism of action of DIM has not been investigated in androgen receptor (AR)–positive hormone-responsive and -nonresponsive prostate cancer cells. Therefore, we investigated the effects of B-DIM, a formulated DIM with greater bioavailability, on AR, Akt, and nuclear factor κB (NF-κB) signaling in hormone-sensitive LNCaP (AR+) and hormone-insensitive C4-2B (AR+) prostate cancer cells. We found that B-DIM significantly inhibited cell proliferation and induced apoptosis in both cell lines. By Akt gene transfection, reverse transcription-PCR, Western blot analysis, and electrophoretic mobility shift assay, we found a potential crosstalk between Akt, NF-κB, and AR. Importantly, B-DIM significantly inhibited Akt activation, NF-κB DNA binding activity, AR phosphorylation, and the expressions of AR and prostate-specific antigen, suggesting that B-DIM could interrupt the crosstalk. Confocal studies revealed that B-DIM inhibited AR nuclear translocation, leading to the down-regulation of AR target genes. Moreover, B-DIM significantly inhibited C4-2B cell growth in a severe combined immunodeficiency–human model of experimental prostate cancer bone metastasis. These results suggest that B-DIM-induced cell proliferation inhibition and apoptosis induction are partly mediated through the down-regulation of AR, Akt, and NF-κB signaling. These observations provide a rationale for devising novel therapeutic approaches for the treatment of hormone-sensitive, but more importantly, hormone-refractory prostate cancer by using B-DIM alone or in combination with other therapeutics. (Cancer Res 2006; 66(20): 10064-72)

Introduction

Prostate cancer is the most frequently diagnosed cancer and is the leading cause of cancer death in men in the U.S. with an estimated 234,460 new cases and 27,350 deaths in 2006 (1). Despite the initial efficacy of androgen deprivation therapy, most patients with advanced prostate cancer eventually develop resistance to this therapy and progress to hormone-refractory prostate cancer (HRPC), for which there is no curative therapy (2). Chemotherapy for prostate cancer has been used for a number of years (3), however, only limited improvement in survival was recently observed in HRPCs when treated with docetaxel-based combination treatment (4). Therefore, novel targeted therapeutic approaches must be developed for the treatment of HRPC.

During the progression of prostate cancers from androgen-sensitive status to an androgen-independent stage, prostate cancer cells still contain androgen receptor (AR), suggesting that AR signaling plays a critical role in the development and progression of prostate cancer (5). AR is a member of the steroid receptor superfamily and is a nuclear transcription factor. Upon binding to AR, androgen activates AR, which, in turn, interacts with androgen response elements (ARE) in the promoter of target genes including prostate-specific antigen (PSA), regulating the transcription of target genes. PSA is a clinically important marker used to monitor diagnosis, treatment response, prognosis, and progression in patients with prostate cancer (6). In addition to androgen, the activity of AR may be modified by molecules in other cell signaling pathways. It has been reported that Akt and nuclear factor κB (NF-κB) regulate the AR signaling pathway by phosphorylation of AR or transcriptional regulation of AR (7, 8). Akt specifically binds to AR and phosphorylates serines 213 and 791, thereby activating AR (7). Blocking the Akt pathway by a dominant-negative Akt or an inhibitor of Akt abrogates the HER-2/neu-induced AR signaling (7). These results suggest that Akt is an activator of AR required for androgen-independent survival and growth of prostate cancer cells mediated by HER-2/neu signaling. It has been known that there are NF-κB binding sites in the promoter of AR (8), suggesting that NF-κB may regulate the expression of AR. The activation of Akt and NF-κB has been involved in the progression of prostate cancer from androgen dependence to independence (9, 10). In HRPC, promiscuous function of AR, together with the activation of Akt and NF-κB pathways, promotes cancer cells to become resistant to androgen deprivation therapy (9–12). In addition, androgen is also known to produce oxidative stress resulting in the production of reactive oxygen species, that, in turn, activate NF-κB and contribute to the induction of tumor cell proliferation (13). Therefore, AR, Akt, and NF-κB could be potential targets for the treatment of prostate cancer, especially HRPC.

3,3′-Diindolylmethane (DIM), an *in vivo* dimeric product of indole-3-carbinol (I3C), exhibits potent antiproliferative activities

Note: M.M.R. Bhuiyan and Y. Li contributed equally to this work.

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against various cancers including prostate cancer (14–16). We have reported that I3C significantly induced apoptosis and inhibited NF-κB and Akt activation in breast and prostate cancer cells, suggesting that I3C could be a chemopreventive and/or therapeutic agent for breast and prostate cancers (17–19). In addition, DIM has been shown to be an androgen antagonist (20), suggesting that the growth-inhibitory effects of DIM on prostate cancer could be due to the inactivation of multiple signaling pathways including AR signaling. To enhance the effects of DIM, Anderton et al. reported a formulated DIM (B-DIM from BioResponse, Boulder, CO), which showed approximately 50% higher bioavailability *in vivo* (21). However, no studies have been reported to date to elucidate the effect and molecular mechanisms of action of B-DIM on prostate cancer cells, especially on HRPC cells. Moreover, B-DIM is currently undergoing phase I studies in our cancer center; thus, we sought to investigate the molecular effects of B-DIM on paired androgen-sensitive LNCaP and androgen-insensitive C4-2B (derived from LNCaP cells) prostate cancer cell lines. Here, we report that B-DIM is a potent agent for inducing proliferation inhibition and apoptotic cell death of both androgen-sensitive LNCaP and androgen-insensitive C4-2B prostate cancer cells. This effect of B-DIM was partly mediated through the down-regulation of AR, Akt, and NF-κB signaling pathways.

Materials and Methods

Cell lines, reagents, and antibodies. Human prostate cancer cell lines including LNCaP, C4-2B, PC-3, and PC-3 stably transfected with AR were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2 atmosphere at 37°C. B-DIM was generously provided by Dr. Michael Zeligs (BioResponse) and was dissolved in DMSO to make a 50 mmol/L stock solution. Dihydrotestosterone (DHT; Sigma, St. Louis, MO) was dissolved in ethanol to make 100 μmol/L stock solutions. Anti-AR (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pAR Ser^{213} (Imgenex, San Diego, CA), anti-PSA (Lab Vision, Fremont, CA), anti-Akt (Santa Cruz Biotechnology), anti-pAkt Ser^{473} (Cell Signaling, Danvers, MA), anti-NF-κB p65 (Upstate, Charlottesville, VA), and anti-β-actin (Sigma) primary antibodies were used for Western blot analysis or confocal microscopic study.

Cell proliferation inhibition studies by MTT assay. Human LNCaP and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 hours, the cells were treated with 0.1, 1, 10, 25, and 50 μmol/L B-DIM for 48 to 72 hours. Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were incubated with MTT (0.5 mg/mL, Sigma) in medium at 37°C for 2 hours and then with isopropanol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 595 nm.

Histone/DNA ELISA for detection of apoptosis. The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in prostate cancer cells treated with B-DIM according to the manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from LNCaP and C4-2B cells treated with 0, 10, and 25 μmol/L B-DIM for 24, 48, and 72 hours were extracted and incubated in microtiter plate modules coated with antihistone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments, followed by color development with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 405 nm.

Western blot analysis. LNCaP, C4-2B, PC-3, and PC-3 cells stably transfected with AR were cultured in RPMI 1640 with 10% FBS or 10% dextran-coated charcoal-stripped FBS (DCC-FBS). Cells were then treated with B-DIM at various concentrations for different time periods followed by treatment with and without DHT (0.1 and 1 nmol/L) for 2 hours. After treatment, cells were lysed and protein concentrations were then measured using bicinchoninic acid protein assay (Pierce, Rockford, IL). The proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with various primary antibodies, and subsequently incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

PSA concentration detection. LNCaP and C4-2B cells were grown in six-well plates in complete RPMI 1640. When cells were 60% confluent, the monolayers were washed with serum-free medium and maintained in serum-free and phenol red–free RPMI 1640 with or without 25 μmol/L of B-DIM treatment for 24 and 48 hours. The conditioned medium was then collected and the protein concentration in the conditioned medium was quantified. The conditioned medium with equal amounts of protein for each sample was subjected to PSA detection using Human PSA ELISA Kit (Anogen, Mississauga, Ontario, Canada) according to the manufacturer's protocol.

Real-time reverse transcription-PCR analysis. LNCaP, C4-2B, and AR stably transfected PC-3 cells were treated as described above. Total RNA was extracted using Trizol (Invitrogen) and purified by using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (2 μg) from each sample was subjected to reverse transcription using the SuperScript First-Strand cDNA Synthesis Kit (Invitrogen) and the cDNAs were subjected to real-time PCR analysis for AR and PSA expression. Real-time PCR reactions were carried out in SmartCycler II (Cepheid, Sunnyvale, CA). The primers for AR were as follows: 5′-AGCCATTGAGCCAGGTGTAG-3′ and 5′-CGTGTAAGTTG-CGGAAGCC-3′. The primers for PSA were as follows: 5′-GTGGGTC-CCGGTTGTCT-3′ and 5′-AGCCCAGCTCCCTGTCT-3′. PCR amplification efficiency and linearity for each gene including targeted and control genes were tested. Data was analyzed according to the comparative cycle threshold (Ct) method and were normalized by β-actin or glyceraldehyde-3-phosphate dehydrogenase expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

NF-κB DNA-binding activity measurement. LNCaP and C4-2B cells were treated as described above. Nuclear extracts were prepared according to the method described by Chaturvedi et al. (22), and the protein concentration was measured. Nuclear protein was then subjected to electrophoretic mobility shift assay (EMSA). EMSA was done by incubating 4 μg of nuclear proteins with IRDye-700 labeled NF-κB oligonucleotide and 2 μg of poly(dI-dC) for 30 minutes at room temperature in the dark. The DNA-protein complex formed was separated from free oligonucleotide on an 8% native polyacrylamide gel followed by scanning with the Odyssey Imaging System (LI-COR, Lincoln, NE).

Immunofluorescence staining and confocal imaging. NCaP and C4-2B cells were plated on coverslips in each well of a six-well plate containing 10% DCC-FBS. Cells were then treated with B-DIM (10 and 25 μmol/L) for different time periods followed by incubation with and without DHT (0.1 and 1 nmol/L) for 2 hours. Cells were then fixed with 10% formalin for 10 minutes. Then, coverslips were rinsed with PBS, treated with 0.2% bovine serum albumin in PBS for 45 minutes and with 0.5% Triton X-100 in PBS for 10 minutes, and incubated with anti-AR monoclonal antibody (1:50; Santa Cruz Biotechnology) at 37°C for 2.5 hours in PBS with 0.5% Triton X-100. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse antibody (1:100; Molecular Probes, Eugene, OR) along with 0.1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at 37°C for 1 hour and washed with PBS. Cell images were captured on a Zeiss 310 laser-scanning inverted confocal microscope system, using 63× 1.2 objective and 488/364 nm laser wavelengths to detect FITC and DAPI, respectively.

Transient transfection with Akt cDNAs and/or reporter constructs. pLNCX-Akt (wild-type Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control empty vector) were generously provided by Dr. Sellers (Dana-Farber Cancer Institute, Boston, MA). NF-κB-Luc (Stratagene, La Jolla, CA) contains six repeated copies of the NF-κB DNA-binding site and a luciferase reporter gene. pSV-β-gal reporter vector (Promega, Madison, WI) transfection was used for normalization of transfection efficiency. The pLNCX-Akt, pLNCX-Myr-Akt,

pLNCX-Akt-K179M, or pLNCX was transiently cotransfected with NF-κB-Luc and pSV-β-gal into LNCaP and C4-2B cells using ExGen 500 (Fermentas, Hanover, MD). After 5 hours, the transfected cells were washed and incubated overnight with complete RPMI 1640, followed by treatment with 50 μmol/L of B-DIM for 48 hours. Subsequently, the luciferase activities in the samples were measured by Steady-Glo Luciferase Assay System (Promega) and ULTRA Multifunctional Microplate Reader (Tecan). β-Galactosidase activities were measured using the β-Galactosidase Enzyme Assay System (Promega). The nuclear proteins from transfected cells were also extracted and subjected to measurement of NF-κB DNA-binding activity using the EMSA method described above. The protein expressions of AR, p-AR, PSA, Akt, p-Akt(Ser^{473}), and NF-κB in transfected LNCaP and C4-2B cells treated with or without B-DIM were measured by Western blot analysis.

LNCaP and C4-2B cells were also transiently cotransfected with PSA-Luc promoter construct containing ARE and pSV-β-gal vector by ExGen 500 (Fermentas). PSA-Luc construct was generously provided by Dr. Charles Young (Mayo Clinic, Rochester, MN). After 5 hours, the transfected cells were washed and incubated overnight with complete RPMI 1640 followed by treatment with 10 and 25 μmol/L of B-DIM for 24 and 48 hours. Subsequently, the luciferase and β-galactosidase activities in the samples were measured as described above.

Animal studies. The severe combined immunodeficiency (SCID)-human prostate cancer model of experimental bone metastasis used for our study was described previously (23, 24). Briefly, male homozygous CB-17 *scid/scid* mice, aged 4 weeks, were purchased from Taconic Farms (Germantown, NY). Human male fetal bone tissue was obtained by a third-party, nonprofit organization (Advanced Bioscience Resources, Alameda, CA) and written informed consent was obtained from the donor, consistent with regulations issued by each state involved and the federal government. After 1 week of acclimatization, the mice were implanted with a single human fetal bone fragment as described previously (23, 24). Suspensions of C4-2B cells (1×10^6 cells in a volume of 20 μL of RPMI 1640) were injected intraosseously by insertion of a 27-gauge needle through the mouse skin directly into the marrow surface of the previously implanted bone. The mice were divided into two groups: control and B-DIM treatment groups. In the B-DIM treatment group, the mice were treated with B-DIM (1 mg/d) by gavage every day for a total of 7 weeks. The volume of the bone tumor in each group was determined by weekly caliper measurements according to the formula $ab^2/2$: where a, length; b, cross-sectional diameter.

Results

Inhibition of cell proliferation by B-DIM. Prior to our molecular experiments, we tested the effect of androgen (DHT) on the proliferation of LNCaP and C4-2B cells. As expected, LNCaP cells, which are known to be sensitive to androgen, showed growth stimulation of 25% and 35% at 48 and 72 hours, respectively, after treatment with 1 nmol/L of DHT. In contrast, C4-2B cells, which are insensitive to androgen, showed minimal or no growth stimulation (data not shown). In our pilot studies, we tested the effects of DIM and B-DIM on cell growth and found no significant difference at similar concentrations *in vitro* (data not shown). However, because B-DIM is known to have 50% greater bioavailability *in vivo* and no molecular mechanism, especially in HRPC cells, has been determined, we tested the effects of B-DIM on cell proliferation in prostate cancer cells by MTT assay. We found that the treatment of LNCaP and C4-2B prostate cancer cells with B-DIM resulted in a dose- and time-dependent inhibition of cell proliferation (Fig. 1*A* and *B*) with maximal inhibition seen at 50 μmol/L, demonstrating a potent growth-inhibitory effect of B-DIM on both androgen-sensitive and -insensitive prostate cancer cells. Because the inhibition of cell proliferation by B-DIM could also be due to the induction of apoptosis, we next tested the apoptosis-inducing effects of B-DIM in prostate cancer cells.

Induction of apoptosis by B-DIM in prostate cancer cells. To investigate whether the growth-inhibitory effect of B-DIM is due to the induction of apoptosis, a histone/DNA ELISA was conducted in B-DIM-treated LNCaP and C4-2B prostate cancer cells. We observed a significant induction of apoptosis in both androgen-dependent and -independent prostate cancer cells (Fig. 1*C* and *D*). This induction of apoptosis was time-dependent and directly correlated with the inhibition of cell proliferation, suggesting that the growth-inhibitory activity of B-DIM was partly mediated through an increase in apoptotic cell death. These results are in direct agreement with those observed earlier in I3C-treated breast and prostate cancer cells (17–19). Because AR and PSA play critical roles in the initiation and progression of prostate cancer, we

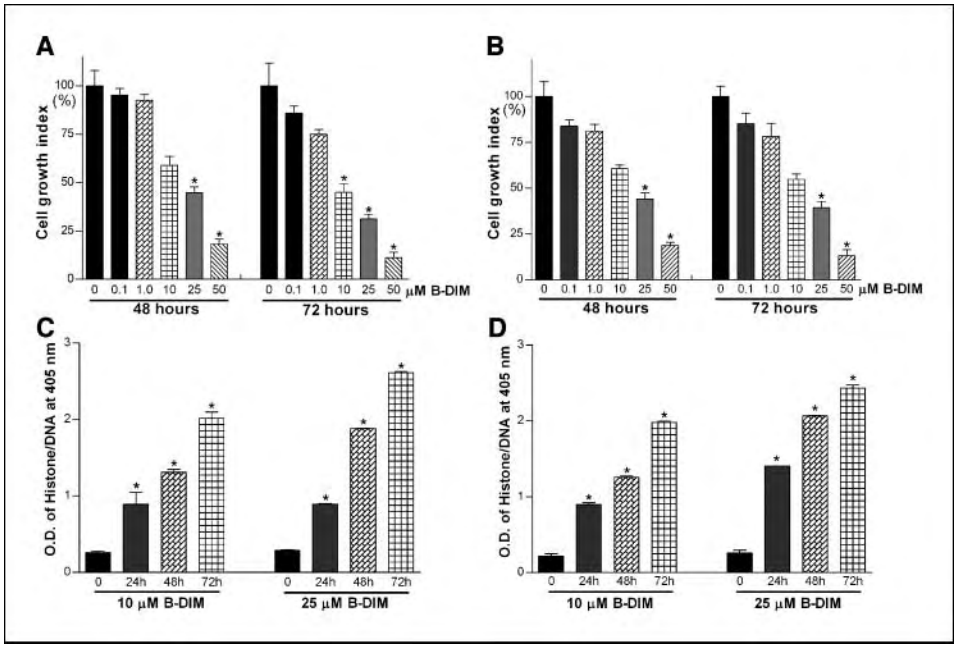

Figure 1. B-DIM significantly inhibited cell proliferation and induced apoptosis in LNCaP and C4-2B cells. For cell proliferation inhibition assays, LNCaP (*A*) and C4-2B (*B*) cells were treated with 0 to 50 μmol/L of B-DIM for 48 and 72 hours. Cell viability was tested by MTT assay. For apoptosis assay, LNCaP (*C*) and C4-2B (*D*) cells were treated with 10 or 25 μmol/L B-DIM for 24, 48, and 72 hours. Apoptotic cell death was tested by histone/DNA ELISA. *, $P < 0.05$ compared with control ($n = 3$).

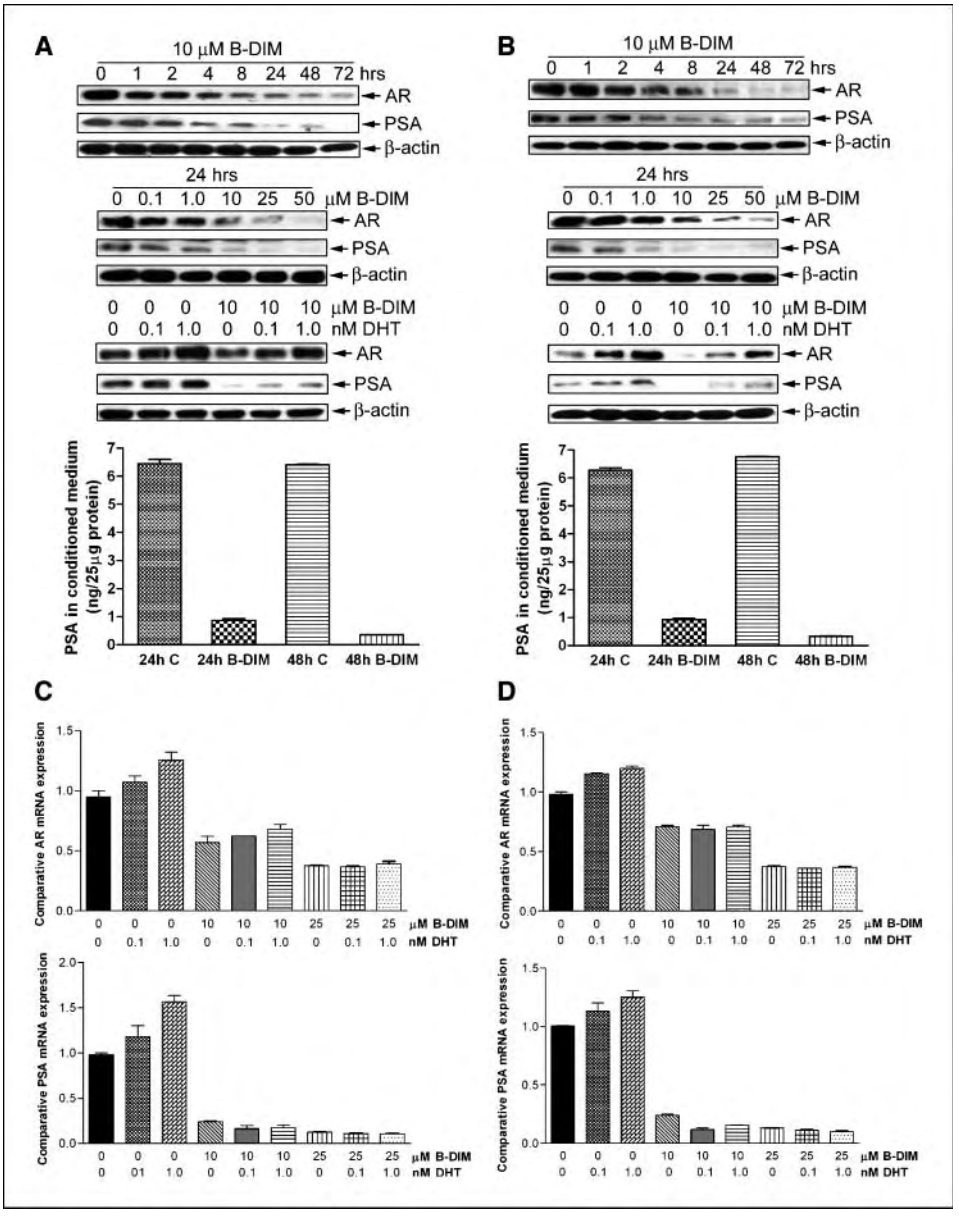

Figure 2. B-DIM significantly inhibited the expression of AR and PSA at mRNA and protein levels and the secretion of PSA in LNCaP (*A* and *C*) and C4-2B (*B* and *D*) cells. Western blot analysis showed that B-DIM inhibited the expression of AR and PSA protein in a time- and dose-dependent manner and that 10 μmol/L of B-DIM abrogated the induction of AR and PSA expression induced by 0.1 and 1 nmol/L of DHT treatment for 2 hours in LNCaP (*A*) and C4-2B (*B*) cells. PSA ELISA showed that 25 μmol/L of B-DIM significantly inhibited the secretion of PSA in LNCaP (*A*) and C4-2B (*B*) cells. Real-time reverse transcription-PCR showed that 10 and 25 μmol/L of B-DIM inhibited AR and PSA mRNA expressions stimulated by 0.1 and 1 nmol/L of DHT in LNCaP (*C*) and C4-2B (*D*) cells.

detected the effects of B-DIM on AR and PSA expression by reverse transcription-PCR and Western blot analysis.

Inhibition of AR and PSA expressions in prostate cancer cells. By Western blot analysis, we found that B-DIM significantly inhibited the expression levels of AR and PSA proteins, as well as the secretion of PSA in LNCaP prostate cancer cells in both time-dependent and dose-dependent manners (Fig. 2*A*). Similarly, B-DIM also showed significant time-dependent and dose-dependent inhibition of AR and PSA protein expression and PSA secretion in C4-2B prostate cancer cells (Fig. 2*B*). Furthermore, B-DIM abrogated the DHT-induced up-regulation of AR and PSA proteins in both LNCaP (Fig. 2*A*) and C4-2B cells (Fig. 2*B*).

In order to further investigate whether down-regulation of AR and PSA proteins by B-DIM is a transcriptional event, we examined the expression levels of both AR and PSA mRNA by real-time reverse transcription-PCR. The results showed that B-DIM significantly down-regulated the expression of both AR and PSA mRNA in the presence and absence of DHT in both LNCaP (Fig. 2*C*) and C4-2B cells (Fig. 2*D*), indicating that B-DIM could inhibit the basal and DHT-induced AR and PSA transcriptional activities. These results are consistent with B-DIM-regulated AR and PSA expression at the protein level, suggesting that B-DIM-regulated AR and PSA gene expressions are transcriptional events.

In order to further prove this point, we tested the effects of B-DIM on AR stable clones of PC-3 prostate cancer cells in which the expression of AR is driven by an artificial promoter. We did not find down-regulation of AR by B-DIM in AR stably transfected PC-3 cells (Fig. 3*A*). We also transfected PSA-Luc vector, which contains PSA promoter with ARE and luciferase reporter gene, into LNCaP and C4-2B cells and treated the cells with B-DIM. We found that luciferase activity was significantly induced after PSA-Luc transfection and 10 to 25 μmol/L of B-DIM treatment for 24 to 48 hours significantly decreased luciferase activity in PSA-Luc-transfected LNCaP and C4-2B cells (Fig. 3*B* and *C*). These results further showed that the down-regulation of AR and PSA expression by B-DIM is a transcriptional event. Because it has been reported that

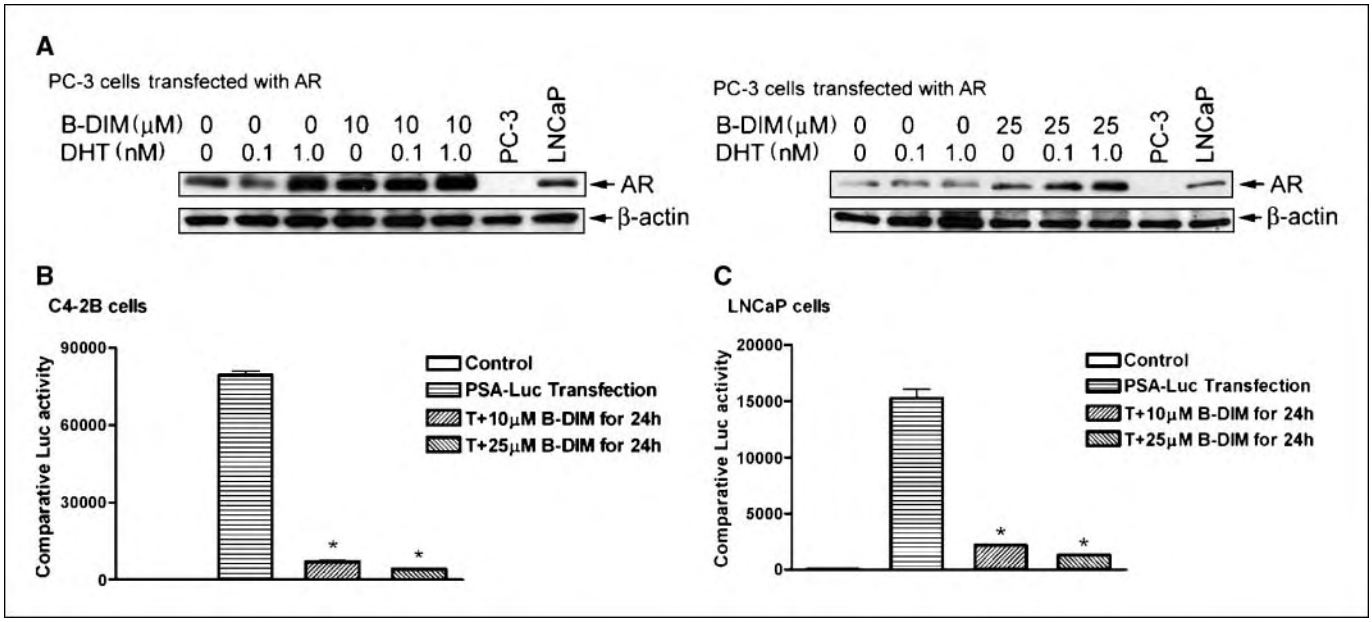

Figure 3. B-DIM did not inhibit the expression of AR in AR stably transfected PC-3 cells, but it significantly inhibited the activity of PSA promoter in LNCaP and C4-2B cells. *A,* B-DIM did not inhibit AR protein expression in AR stably transfected PC-3 cells. AR stably transfected PC-3 cells were treated with 10 and 25 μmol/L of B-DIM for 24 hours followed by 0.1 and 1 nmol/L of DHT treatment for 2 hours. Western blot analysis showed that both 10 and 25 μmol/L of B-DIM did not inhibit the AR expression stimulated by DHT. C4-2B (*B*) and LNCaP (*C*) cells were transiently cotransfected with PSA-Luc and pSV-β-gal by ExGen 500. After 5 hours, the transfected cells were washed and incubated overnight with complete RPMI 1640 followed by treatment with 10 and 25 μmol/L of B-DIM for 24 hours. Subsequently, the luciferase and β-galactosidase activities in the samples were measured. The comparative luciferase activity was calculated and adjusted by β-galactosidase activity. *, $P < 0.05$ compared with PSA-Luc transfection ($n = 3$).

AR may be regulated by Akt and NF-κB (7, 8), we next tested the effects of B-DIM on Akt and NF-κB signaling.

Down-regulation of Akt by B-DIM leading to inhibition of NF-κB, AR, and PSA. By Western blot analysis, we found that B-DIM down-regulated the protein levels of p-Akt, nuclear NF-κB, AR, p-AR, and PSA in both LNCaP and C4-2B cells (Fig. 4). To further investigate the relationship between Akt, NF-κB, AR, and PSA, and the effects of B-DIM on these molecules, we cotransfected Akt cDNA and NF-κB-Luc into LNCaP and C4-2B cells. We found that p-Akt, nuclear NF-κB, AR, p-AR(Ser^{213}), and PSA were up-regulated after wild-type Akt and Myr Akt transfections in LNCaP and C4-2B cells. However, the up-regulation of p-Akt, nuclear NF-κB, AR, p-AR(Ser^{213}), and PSA by Akt transfection were significantly abrogated in both LNCaP and C4-2B cells (Fig. 4).

Using transfection and luciferase assays, we found that luciferase activity was significantly increased after cotransfection with NF-κB-Luc and wild-type Akt or Myr Akt in both LNCaP and C4-2B cells (Fig. 5*A*), suggesting the activation of NF-κB by Akt transfection. Moreover, B-DIM treatment significantly abrogated the up-regulation of luciferase activity caused by Akt transfection. Furthermore, we conducted EMSA to test NF-κB DNA binding activity in Akt-transfected cells. The results showed that NF-κB DNA binding activity was significantly increased by wild-type Akt and Myr Akt transfection and this was inhibited by B-DIM treatment (Fig. 5*B*), which is consistent with the data from luciferase assays. Moreover, we also found that B-DIM at 10 or 25 μmol/L significantly inhibited NF-κB DNA binding activity in the presence and absence of DHT in both LNCaP and C4-2B cells (Fig. 5*C*).

Combined with the alteration in AR as observed by Akt transfection studies, our results suggest that there could be a crosstalk between p-Akt, NF-κB, and AR, and that the inhibition of Akt activation by B-DIM could lead to the down-regulation of NF-κB, AR, and PSA. Because the functions of AR in the regulation of its target genes mainly occur in the nucleus, we next tested the localization of AR before and after B-DIM treatment.

Inhibition of AR nuclear translocation by B-DIM. By Western blot analysis, we found that B-DIM reduced AR protein levels in both the cytosol and nuclear extracts (Fig. 4). However, the AR protein level was much more down-regulated by B-DIM in the nucleus than in the cytosol, suggesting that B-DIM could inhibit AR nuclear translocation. Therefore, we conducted immunofluorescent staining and confocal imaging to examine the effect of B-DIM on AR nuclear translocation in LNCaP and C4-2B prostate cancer cells. We found that both LNCaP and C4-2B cells treated with 25 μmol/L of B-DIM for 24 hours showed much less AR staining in the nucleus compared with control cells (Fig. 6*A* and *B*). These results suggest that B-DIM significantly inhibited AR translocation into the nucleus and, in turn, down-regulated AR target genes including PSA, consistent with reverse transcription-PCR and Western blot data showing the down-regulation of PSA mRNA and protein levels after B-DIM treatment (Figs. 2 and 4).

Inhibition of C4-2B bone tumor growth *in vivo* by B-DIM. To test whether B-DIM has any antitumor activity *in vivo*, we conducted a pilot animal experiment as "proof-of-concept" using a SCID-human model of experimental prostate cancer bone metastasis. After injection of C4-2B prostate cancer cells into fetal bone that was previously implanted, we found significant tumor growth, and the bone was severely destroyed by the infiltrating prostate cancer cells as observed previously (24); however, the residual bone also showed active new bone formation (Fig. 6*C*). Importantly, we found that B-DIM significantly inhibited C4-2B bone tumor growth in this model of prostate cancer bone metastasis, demonstrating the inhibitory effect of B-DIM on

prostate cancer bone metastasis (Fig. 6*C*). The body weight of mice in each group did not show any significant difference, suggesting no obvious toxicity due to B-DIM treatment.

Discussion

Androgen exerts its biological effects by binding to AR and activating AR transcriptional activity, promoting the growth of prostate epithelial cells. Therefore, androgen and AR play important roles in the normal development and maintenance of the prostate (5). More importantly, AR signaling also plays critical roles in the development and progression of prostate cancer. In the

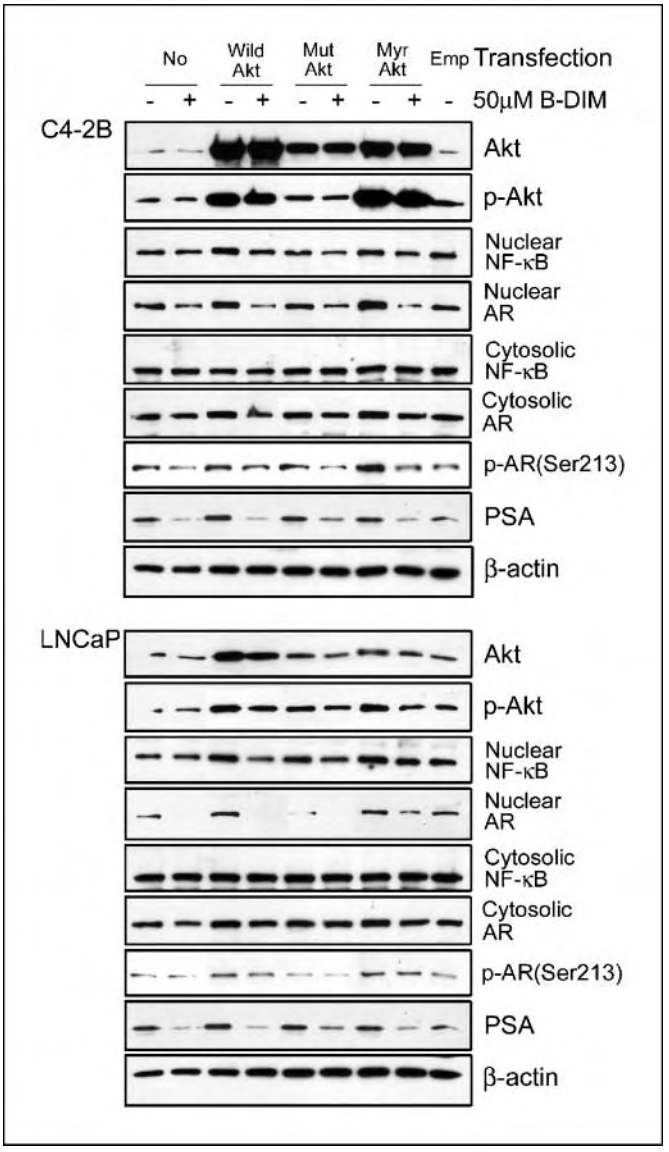

Figure 4. B-DIM inhibited the expression of p-Akt, nuclear NF-κB, AR, and PSA in Akt transfected C4-2B and LNCaP cells. C4-2B and LNCaP cells were transiently cotransfected with NF-κB-Luc and pLNCX-Akt (wild-type Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control empty vector). After 5 hours, the transfected cells were washed and incubated overnight with complete RPMI 1640 followed by treatment with 50 μmol/L of B-DIM for 48 hours. Subsequently, the cells were lysed and Western blot analysis was conducted to test the expression of Akt, p-Akt, AR, PSA, and NF-κB. *Wild Akt,* wild-type Akt; *Mut Akt,* dominant-negative Akt; *Myr Akt,* constitutively activated Akt; *Emp,* control empty vector.

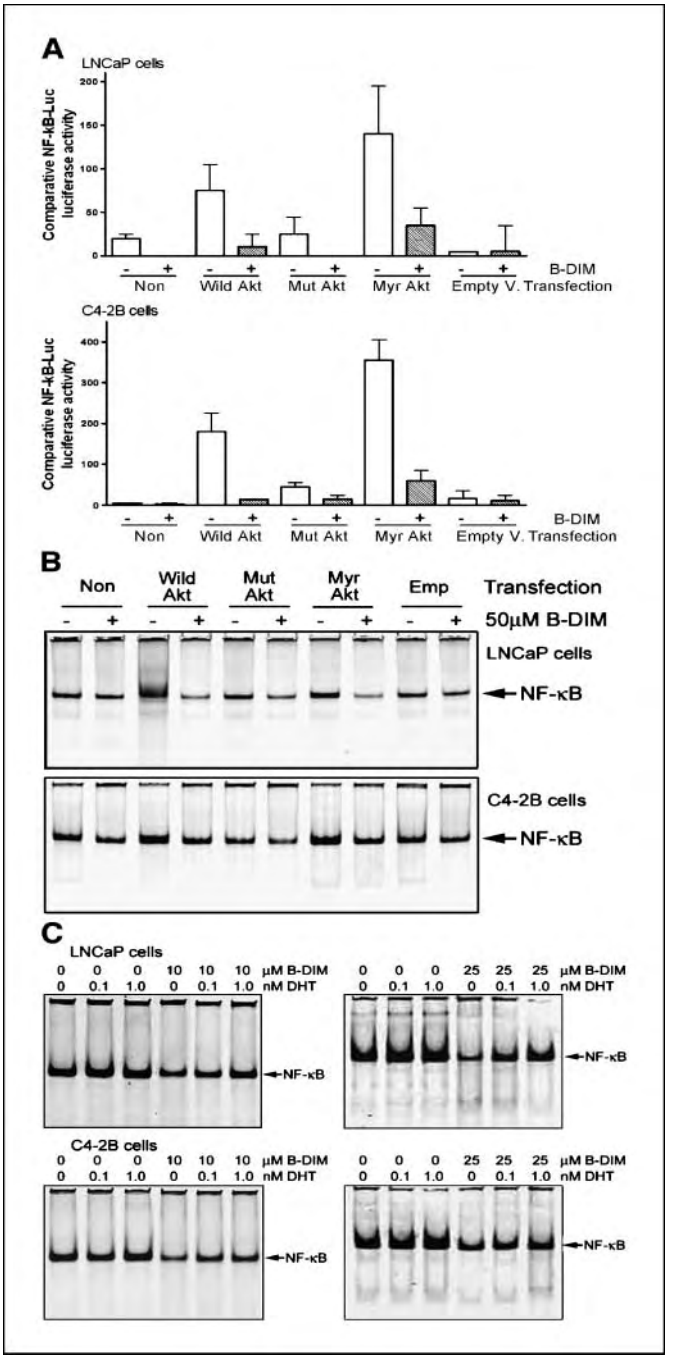

Figure 5. B-DIM inhibited NF-κB DNA binding activity in C4-2B and LNCaP cells. *A,* B-DIM inhibited NF-κB DNA binding activity in Akt transfected C4-2B and LNCaP cells tested by luciferase assay. C4-2B and LNCaP cells were transiently cotransfected with NF-κB-Luc and pLNCX-Akt (wild-type Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control empty vector). After 5 hours, the transfected cells were washed and incubated overnight with complete RPMI 1640 followed by treatment with 50 μmol/L of B-DIM for 48 hours. Subsequently, the luciferase activity in the transfected cells was tested by luciferase assay, *Wild Akt,* wild-type Akt; *Mut Akt,* dominant negative Akt; *Myr Akt,* constitutively activated Akt; *Emp,* control empty vector. *B,* B-DIM inhibited NF-κB DNA binding activity in Akt transfected C4-2B and LNCaP cells tested by EMSA. *C,* B-DIM significantly inhibited NF-κB DNA binding activity stimulated by DHT in LNCaP and C4-2B cells. LNCaP and C4-2B cells were treated with 10 and 25 μmol/L of B-DIM for 24 hours followed by 0.1 and 1 nmol/L of DHT treatment for 2 hours. Nuclear protein was extracted. EMSA showed that both 10 and 25 μmol/L of B-DIM abrogated the activation of NF-κB DNA binding activity stimulated by DHT in LNCaP and C4-2B cells.

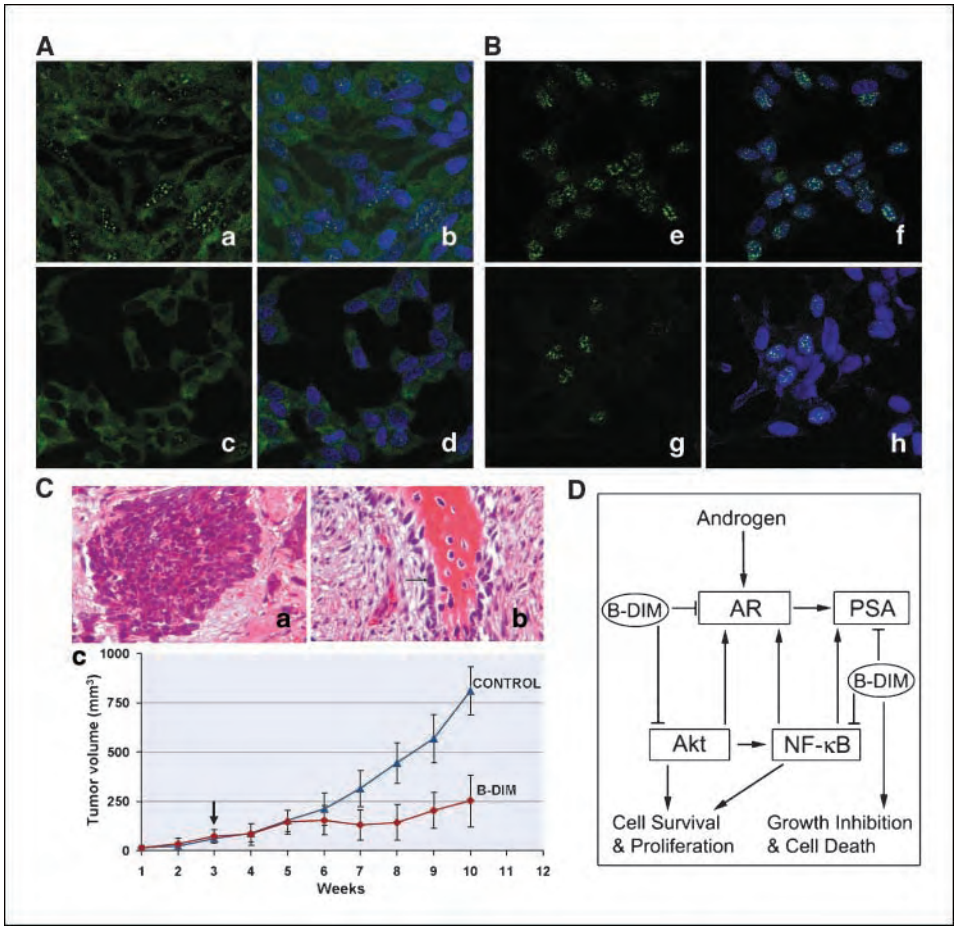

Figure 6. B-DIM significantly inhibited AR nuclear translocation in LNCaP and C4-2B cells. LNCaP (*A*) and C4-2B cells (*B*) were treated with 25 μmol/L of B-DIM for 24 hours followed by 1 nmol/L of DHT treatment for 2 hours. Cells were then incubated with anti-AR monoclonal antibody followed by incubation with FITC-conjugated anti-mouse antibody along with 0.1 μg/mL of DAPI. Cell images were captured on a confocal microscope system, using 63× 1.2 objective and 488/364 nm laser wavelengths to detect FITC and DAPI, respectively. *a* and *b,* LNCaP cell control; *c* and *d,* LnCaP cells treated with 25 μmol/L of B-DIM; *e* and *f,* C4-2B cell control; *g* and *h,* C4-2B cells treated with 25 μmol/L of B-DIM. *C,* B-DIM significantly inhibited C4-2B prostate cancer cell growth in SCID-human model of prostate cancer bone metastasis. *a,* tumor cells forming large sheets; *b,* active osteoblasts lining up on the surface of bone matrix surface; *c,* tumor size in B-DIM treatment group was smaller compared with control ($P < 0.05$, $n = 4$; *arrow,* start of B-DIM treatment). *D,* crosstalk of Akt, NF-κB, and PSA with AR and the effects of B-DIM on the crosstalk.

early stage of prostate cancer, androgen deprivation therapy shows inhibition of cancer cell growth, suggesting that androgen still controls functions of AR signaling at this stage. However, in HRPC, androgen deprivation therapy fails, although AR expression is still maintained in most cases (5). Actually, 30% of HRPCs show amplification and overexpression of the AR gene, indicating that the AR signaling pathway is malfunctioning in HRPC (25, 26). Studies on the progression of prostate cancer have indicated that an increase in AR mRNA and protein is both necessary and sufficient to convert prostate cancer from a hormone-sensitive to a hormone-refractory stage (27), and that overexpressed AR linked to $p21^{waf1}$ silencing may be responsible for androgen independence and resistance to apoptosis (28). These studies suggest that overexpression of AR is an important factor for acquired resistance of prostate cancer to androgen deprivation therapy. Therefore, AR is a key target for the treatment of both early stage prostate cancer and HRPC, and the inactivation of AR expression should be an important approach for the successful treatment of HRPC. In this study, we found that B-DIM significantly inhibited cell proliferation, induced apoptosis, down-regulated the expression of AR mRNA and protein, and abrogated the activation of AR by DHT in both androgen-sensitive and -insensitive prostate cancer cells. These results suggest that cell proliferation inhibition and induction of apoptosis by B-DIM is partly mediated by the down-regulation of AR. Although the degree of alteration of AR expression is higher than that of cell growth inhibition by B-DIM at the same time point, we believe that these phenomena could be due to the fact that B-DIM may have caused alterations of AR and other gene expressions before we observed changes in the biological behaviors of cells such as cell proliferation. B-DIM also inhibited AR nuclear translocation and subsequent expression and secretion of PSA, one of the important markers for progression of prostate cancer. Moreover, B-DIM significantly inhibited C4-2B prostate cancer cell growth in the implanted bone in SCID-human models of prostate cancer bone metastasis. These results suggest that B-DIM could be a potent agent for the treatment of androgen-sensitive, but most importantly, androgen-insensitive prostate cancer.

Several cell signal transduction pathways have been involved in the progression of HRPC by the interaction with AR signaling (29–31). Among them, the Akt pathway is an important cell signaling pathway for the survival of prostate cancer cells (32). It is believed that increased AR activity is caused by a crosstalk between AR, phosphoinositide-3-kinase (PI3K)/Akt, and mitogen-activated protein kinase pathways (29), although the relationship between Akt and AR remains controversial. It has been reported that Akt directly phosphorylates AR at Ser^{213} or indirectly interacts with AR through GSK3β and β-catenin, and then enhances AR transactivation, promoting the growth of prostate cancer cells (7, 30, 33). However, a recent study by Yang et al. showed that inhibition of the PI3K/Akt pathway by LY294002 could result in the activation of FOXO3a, which could then induce AR expression to protect prostate cancer cells from apoptosis caused by the inhibition of the PI3K/Akt pathway (34). Therefore,

we believe that the inhibition of both Akt and AR signaling pathways could be a powerful approach for the treatment of both androgen-dependent prostate cancer and HRPC. In this study, we found that transfection of Akt caused the activation of NF-κB and increased AR expression and phosphorylation, suggesting that there could be a direct crosstalk between Akt, NF-κB, and AR. We also found that B-DIM inhibited both Akt activation and AR transactivation, suggesting that B-DIM could be a potent agent for the treatment of both androgen-dependent prostate cancer and HRPC.

It has been well known that the NF-κB pathway plays an important role in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling. The NF-κB signaling pathway is also involved in the development and progression of prostate cancer. NF-κB is overexpressed in prostatic intraepithelial neoplasia and prostate adenocarcinoma (35). Constitutive activation of NF-κB has been found in androgen-independent prostate cancer cells, whereas less activity of NF-κB has been observed in androgen-dependent prostate cancer cells (36, 37). Like Akt and AR, the relationship between NF-κB and AR activation remains controversial. Palvimo et al. reported that elevated expression of NF-κB p65 repressed AR-mediated transactivation in a dose-dependent manner, whereas NF-κB p50 did not influence AR transactivation (38). However, other investigators show that IL-4-induced NF-κB is required for AR activation (31), and that there are NF-κB binding sites in the promoter of AR (8), suggesting that the activation of NF-κB could enhance AR transactivation. Therefore, inhibition of both NF-κB and AR could be another powerful approach to treat both androgen-dependent prostate cancer and HRPC. Indeed, in this study, we found that B-DIM inhibited NF-κB, AR, and PSA, resulting in the cell proliferation inhibition and apoptotic cell death in both androgen-sensitive and -insensitive prostate cancer cells. It has been reported that NF-κB activates PSA expression by direct binding to the enhancer of PSA (39). Therefore, the inhibition of PSA expression and secretion by B-DIM could be mediated through the down-regulation of both NF-κB and AR.

In HRPC, the failure of androgen deprivation therapy is believed to be due to the AR modifications including AR mutations, AR amplification, and ligand-independent activation of AR through crosstalk with other signaling pathways (25, 26, 40, 41). Growing evidence shows the critical role of AR activation by nonandrogens in the development of androgen-independent prostate cancer (7, 29–31). Therefore, it is important to discover other nonandrogen molecules which activate AR. By Akt transfection, we observed increased AR expression and phosphorylation accompanied with increased p-Akt, NF-κB, and PSA, suggesting a crosstalk between Akt, NF-κB, AR, and PSA (Fig. 6*D*). It has been reported that Akt regulates NF-κB activation through IKK phosphorylation (42) and that NF-κB may activate AR signaling (31). Therefore, we believe that in the crosstalk, p-Akt could activate NF-κB and, in turn, activate AR which transactivates PSA expression, promoting the growth of prostate cancer cells. In addition, AR could also be activated directly by activated Akt (7), whereas PSA could be up-regulated directly by NF-κB (39). More importantly, we found that B-DIM could inhibit Akt, NF-κB, AR, p-AR, and PSA in both androgen-sensitive and -insensitive prostate cancer cells, demonstrating its effects on the interruption of these crosstalks. Taken together, these results along with our findings on the inhibition of cell growth *in vitro*, antitumor activity *in vivo*, and the induction of apoptosis by B-DIM in both LNCaP and C4-2B prostate cancer cells, clearly suggest that B-DIM could be a promising nontoxic agent for the treatment of prostate cancer, especially HRPC, for which there is no curative therapy. However, further in-depth studies, including further animal experiments and clinical trials, are needed to fully appreciate the value of B-DIM in the fight against prostate cancer.

Acknowledgments

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35 Plant-Derived Antioxidants

Fazlul H. Sarkar and Yiwei Li

1. Introduction

As humans live in an aerobic environment, their exposure to reactive oxygen species (ROS) is continuous and unavoidable. The biological systems in the human body interact with the external environment to maintain an internal environment that favors survival, growth, differentiation, and reproduction. Although a number of defense systems have evolved to combat the accumulation of ROS, these defense systems are not always adequate to counteract the production of ROS, resulting in a state of oxidative stress.[1] It is important to note that oxidative stress has been linked to aging and a variety of chronic diseases such as atherosclerosis, neurodegenerative diseases, diabetes, pulmonary fibrosis, arthritis.[2,3] More importantly, oxidative stress could be carcinogenic because ROS can cause severe DNA damage, which plays an important role in carcinogenesis.[2,4] Once DNA damage occurs, DNA repair is a critical process in order to prevent mutagenesis. However, under oxidative stress, the repair of DNA damage can be inhibited by several redox-dependent metals, resulting in carcinogenesis.[4] Moreover, the activation of nuclear factor-kappa B (NF-κB) by ROS under oxidative stress has been known as a key event in carcinogenesis.[4] Therefore, antioxidants are important in combating cancers and some chronic diseases, which have been tightly linked with oxidative stress.

In nature, to resist oxygenic threat, antioxidants have evolved in parallel with our oxygenic atmosphere. Plants employ antioxidants to defend their structures against ROS produced during photosynthesis.[5] Plants, therefore,

produce various antioxidant components, which could be beneficial for human health. A variety of plant-derived components have been found to reduce oxidative stress via the antioxidant mechanism. Among them, isoflavones, curcumin, epigallocatechin-3-gallate, indole-3-carbinol (I3C), resveratrol, lycopene, vitamin E, and vitamin C have shown more promising effects on the reduction of oxidative stress.[6–12] Most of them have been found to inhibit NF-κB activation stimulated by ROS.[13–20] Moreover, these antioxidants have shown their inhibitory effects on atherosclerosis, neurodegeneration, oncogenesis, cancer growth, and metastasis,[21–24] suggesting that they could be used as chemopreventive and/or chemotherapeutic agents for some chronic diseases and cancers.

2. Oxidative Stress and NF-κB Activation in Chronic Diseases and Cancers

It has been well known that NF-κB activation stimulated by ROS is a very important event in the development of some chronic diseases and cancers,[25,26] which are linked with oxidative stress. Under the situation of oxidative stress, ROS induces DNA damage and alters cell signal transduction pathways including the NF-κB pathway.[27] The direct addition of H_2O_2 to culture medium activates NF-κB in many types of cell lines.[28] In addition, it has been found that ROS in cells is increased in response to the agents that also activate NF-κB.[28,29] These findings suggest that oxidative stress activates NF-κB activity in cells.

NF-κB plays important roles in the physiological processes as well as in the defensive response to injury, infection, and other stress conditions.[30] The NF-κB family is composed of several proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), each of which may form homo- or heterodimers.[31,32] In human cells without specific extracellular signal, NF-κB is sequestered in the cytoplasm through tight association with its inhibitors: IκB, which acts as NF-κB inhibitor, and p100 proteins, which serve as both inhibitors and precursors of NF-κB DNA-binding subunits.[31,33] NF-κB can be activated by many types of stimuli including tumor necrosis factor-α (TNF-α), ultraviolet radiation, H_2O_2, free radicals, etc. The activation of NF-κB occurs through phosphorylation of IκB by

IKKβ and/or phosphorylation of p100 by IKKα, leading to degradation of IκB and/or the processing of p100 into a small form (p52). This process allows two forms of activated NF-κB (p50–p65 and p52–RelB) to become free, translocate into nucleus, bind to NF-κB-specific DNA-binding sites, and regulate downstream gene transcription.[33,34]

In this way, NF-κB controls the expression of many genes that are involved in cellular physiological processes including stress response, inflammation, differentiation, cell growth, apoptosis, etc.[35–37] The disorder of these physiological processes has been demonstrated to be linked with the occurrence of some chronic diseases and cancers. It has been reported that overexpression of NF-κB protects cells from apoptosis and favors cell survival, while inhibition or absence of NF-κB induces apoptosis.[38] An *in vivo* study showed that mice lacking NF-κB p65 died embryonically from extensive apoptosis in the liver, suggesting the anti-apoptotic role of NF-κB.[39] The deregulated cell proliferation or inability of cells to undergo apoptotic cell death results in the development of cancers. NF-κB also promotes the expression of genes related to inflammation and degeneration, resulting in chronic inflammatory and degenerative diseases.[25,40,41] Therefore, the deregulated NF-κB under oxidative stress has been described as a major cause in cancers and some of the chronic diseases.[26,40]

3. NF-κB as a Preventive or Therapeutic Target in Inflammatory Diseases and Cancers

Inhibition of NF-κB activation stimulated by ROS is now widely recognized as a valid strategy to combat inflammatory disease.[25,42] However, it has become obvious that inhibition of NF-κB activity is not only desirable for the treatment of inflammation but also in cancer therapy.[43,44] Examination of the inflammatory microenvironment in neoplastic tissues has supported the hypothesis that inflammation is a cofactor in oncogenesis for a variety of cancers. Many anti-inflammation drugs and antioxidants inhibit NF-κB activity and induce apoptosis; therefore, they may also be desirable in the treatment of cancers.

Experimental studies have shown the cellular growth and anti-apoptotic activity of NF-κB in malignant cells.[45,46] It has been reported that NF-κB

is constitutively activated in Hodgkin's tumor cells, whereas inhibition of NF-κB blocks the cell growth.[46] It has been demonstrated that NF-κB regulates growth and survival of multiple myeloma and that NF-κB is a novel therapeutic target in multiple myeloma.[47] Our data also showed that plant-derived antioxidant compounds including genistein, I3C, and 3,3′-diindolylmethane (DIM) inhibited the activity of NF-κB and the growth of cancer cells, and induced apoptosis in cancer cells,[13,17,48] suggesting that NF-κB is a target for cancer prevention and/or treatment.

Now it has become more obvious that inhibition of NF-κB activity is desirable in the prevention and treatment of cancers and inflammations. Thus, plant-derived antioxidants with NF-κB inactivation activity may serve as agents against cancers and some chronic diseases.

4. Plant-Derived Antioxidants Inhibiting Oxidative Stress and NF-κB Activation

4.1. *Isoflavones*

Isoflavones are a subclass of the more ubiquitous flavonoids and are much more narrowly distributed in soybeans. Genistein, daidzein, and glycitein are three main isoflavones found in soybeans. Genistein and daidzein have been found in relatively high concentration in soybeans and most soy-protein products, while much lower amounts of glycitein are present in soybeans. Experimental studies have revealed that isoflavones, particularly genistein, exert antioxidant effects on human cells. It has been reported that genistein protects cells against ROS by scavenging free radicals and reducing the expression of stress-response-related genes.[6,49] Isoflavones also stimulate antioxidant protein gene expression in Caco-2 cells.[50] Sierens *et al.*[51] have found that isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm, suggesting the antioxidant effects of isoflavone. In addition, it has been found that isoflavones and synthetic isoflavone derivatives suppress lipid peroxidation of human high-density lipoproteins, decrease oxidized low-density lipoproteins, and reduced atherosclerotic plaque thickness, suggesting their preventive and therapeutic effects on cardiovascular diseases.[52,53] Kawakami *et al.*[54] have also

reported that soy isoflavones may reduce the risk of some cardiovascular diseases through their radical scavenging function and hypocholesterolemic action. Moreover, it has been demonstrated that genistein inhibits tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate induced hydrogen peroxide production in human polymorphonuclear leukocytes and HL-60 cells, suggesting the inhibitory effect of genistein on carcinogenesis through antioxidant mechanism.[55]

4.1.1. *Inhibition of oxidative stress and NF-κB activation in vitro by soy isoflavone genistein*

Our laboratory has investigated whether genistein treatment could modulate NF-κB DNA binding activity in PC3 and LNCaP prostate cancer cells by electrophoresis mobility shift assay (EMSA). We found that 50 μM genistein treatment for 24–72 h significantly inhibited NF-κB DNA-binding activity in both cell lines.[13] We further investigated whether genistein could block NF-κB induction by oxidative stress inducers, H_2O_2 and TNF-α,[13] both of which have been previously shown to induce NF-κB DNA-binding activity. After treatment with H_2O_2 or TNF-α, we observed an increase in NF-κB DNA-binding activity in prostate cancer cell lines, as expected. However, when the cells were pre-treated with genistein for 24 h prior to stimulation with the inducing agent, genistein abrogated the induction of NF-κB DNA-binding activity elicited by either H_2O_2 or TNF-α. Western blot analysis of nuclear extracts showed similar results.[13] These results demonstrated that genistein not only reduced NF-κB DNA-binding activity in non-stimulated conditions, but inhibited NF-κB activation in cells under oxidative stress condition.

Other investigators also demonstrated similar effect of genistein on NF-κB in different types of cells. Baxa and Yoshimura[54] showed that genistein reduced NF-κB in T lymphoma cells via a caspase-mediated cleavage of IκBα. Tabary *et al.*[57] also found that genistein inhibited constitutive and inducible NF-κB activation and decreased interleukin-8 production in human cystic fibrosis bronchial gland cells. Our *in vitro* data along with results from other investigators suggested that genistein functions as an antioxidant, which could be a potent agent for the inhibition of oxidative stress and the prevention and/or treatment of cancers.

4.1.2. *Inhibition of oxidative stress and NF-κB activation in vivo by soy isoflavones*

Since our *in vitro* results showed inactivation of NF-κB by genistein treatment, we further investigated the effect of isoflavone supplementation on NF-κB activation *in vivo* in human volunteers.[14] The lymphocytes from healthy male subjects were harvested from peripheral blood and cultured for 24 h in the absence and presence of genistein. EMSA revealed that genistein treatment inhibited basal levels of NF-κB DNA-binding activity by 56% and abrogated TNF-α-induced NF-κB activity by 50%.[14] Furthermore, when human volunteers received 50 mg of soy isoflavone supplements (Novasoy™) twice daily for three weeks, TNF-α failed to activate NF-κB activity in lymphocytes harvested from these volunteers, while lymphocytes from these volunteers collected prior to soy isoflavone intervention showed activation of NF-κB DNA-binding activity upon TNF-α treatment *in vitro*.[14]

We further measured the levels of oxidative DNA damage in the blood of the six subjects before and after supplementation with Novasoy™. DNA was isolated from lymphocyte nuclei from the six subjects and analyzed for levels of 5-OHmdU, a modified DNA base that represents the endogenous status of cellular oxidative stress. We found that the mean value of 5-OHmdU was significantly decreased after three weeks of soy supplementation.[14] These results have demonstrated that isoflavone supplementation is very effective in reducing the level of 5-OhmdU, decreasing oxidative damage, and inhibition of NF-κB activation in humans *in vivo*, providing strong evidence that soy isoflavone functions as an antioxidant and that these effects of isoflavone may be responsible for its chemopreventive activity.

4.1.3. *The effects of soy isoflavone genistein on cancer cells*

The effects of isoflavone genistein on cancer cells have been widely studied in various cancer cells. The results from our laboratory and other investigators have revealed that genistein inhibits the growth of various cancer cells including leukemia, lymphoma, neuroblastoma, breast, prostate, lung, gastric, head, and neck cancer cells.[13,58–65] We and other investigators have

also found that genistein induces apoptosis with modulation of expression of genes related to apoptotic processes.[49,61,63–65] Genistein has been shown to regulate the molecules in cell signaling pathways including Akt, NF-κB, MAPK, p53, AR, and ER pathways.[48,62] By microarray and reverse transcriptase-polymerase chain reaction analysis, we have also found that genistein regulates the expression of genes that are critically involved in the control of cell growth, cell cycle, apoptosis, cell signaling transduction, angiogenesis, tumor cell invasion, and metastasis,[66,67] suggesting its pleiotropic effects on cancer cells. These effects make isoflavone a promising agent against oxidative stress, some chronic diseases, and cancers.

4.2. *Indole-3-carbinol and 3,3'-diindolylmethane*

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 vivo* to its dimeric product DIM. Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form several derivatives.[68] DIM is the major derivative and condensation product of I3C and it is also biologically active. The formation of DIM from I3C has been believed to be a likely prerequisite for I3C-induced anti-carcinogenesis. I3C and DIM have been shown to reduce oxidative stress and stimulate antioxidant response element-driven gene expression as antioxidants.[69,70] Furthermore, we and other investigators have found that I3C and DIM inhibit oncogenesis and cancer cell growth, and induce apoptosis in various cancer cells,[9,17,71–73] suggesting that I3C and DIM may serve as potent agents for prevention and/or treatment of cancers.

We have also investigated whether I3C treatment could inhibit NF-κB DNA-binding activity in prostate and breast cancer cells by EMSA.[17,73] Cancer cells were treated with 60 or 100 μmol/l I3C for 48 h or with 20 μg/l TNF-α for 10 min. Nuclear proteins were harvested from samples, incubated in DNA-binding buffer with ^{32}P labeled NF-κB consensus oligonucleotide, and subjected to 8% non-denatured polyacrymide gel. After drying

the gel, autoradiography of the gel showed that TNF-α treatment stimulated NF-κB activation as expected; however, I3C significantly inhibited NF-κB DNA-binding activity in prostate and breast cancer cells, corresponding with the inhibition of cell proliferation and the induction of apoptosis by I3C in prostate and breast cancer cells.[17,73] These results suggest that inhibition of NF-κB activity by I3C may reduce the oxidative stress induced by ROS or TNF-α.

4.3. *Curcumin*

Curcumin is a compound from *Curcuma longa* (tumeric). *C. longa* is a plant widely cultivated in tropical regions of Asia and Central America. Turmeric extract from the rhizomes, commonly called curcuminoids, is mainly composed of curcumin. Curcumin has recently received considerable attention due to its pronounced anti-inflammatory, anti-oxidative, immunomodulating, anti-atherogenic, and anti-carcinogenic activities.[7,74–76]

Curcumin is a potent scavenger of oxygen free radicals such as hydroxyl radical and nitrogen dioxide radical.[77] It has been reported that curcumin inhibits lipid peroxidation in rat brain, liver, and lens, suggesting its antioxidant properties.[78–80] Chuang *et al.*[81] have shown that curcumin inhibits diethylnitrosamine-induced liver inflammation and activation of NF-κB in rats. Curcumin can also protect against inflammation-related changes. Administration of curcumin decreases the level of the prostanoids in alcohol toxicity model, suggesting its protective effects against inflammation.[82] It has been reported that curcumin inhibited IKK, suppressed both constitutive and inducible NF-κB activation, and potentiated TNF-induced apoptosis.[83] Curcumin also showed strong antioxidant and anticancer properties through regulating the expression of genes that require the activation of activator protein 1 and NF-κB.[84] It has been known that curcumin inhibits the growth of cancer cells, induces apoptosis, reduces cell survival signal protein Akt, and regulates the expression of genes related to anti-invasion.[15,85–87] In addition, NF-κB has been implicated in the development of drug resistance in cancer cells. Curcumin has been found to significantly inhibit chemotherapeutic agent doxorubicin-induced NF-κB activation,[88] suggesting its effect on reducing drug resistance and sensitizing cancer cell to chemotherapeutic agents.

4.4. *Epigallocatechin-3-gallate*

Consumption of green tea has been associated with human health including the prevention of cancer and heart disease. Green tea and its constituents have been studied both *in vitro* and *in vivo*. Green tea contains several catechins including epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG). However, EGCG has been believed to be the most potent for inhibition of oncogenesis and reduction of oxidative stress among these catechins.[24,89]

EGCG has been shown to have strong antioxidant activity. It has been reported that EGCG treatment resulted in a significant dose- and time-dependent inhibition of activation and translocation of NF-κB to the nucleus by suppressing the degradation of IκBα in the cytoplasm.[90,91] EGCG has also been shown to inhibit activation of IKK and phosphorylation of IκBα, corresponding with the inhibition of activation of NF-κB.[92,93] There are growing evidences showing that EGCG inhibits the proliferation of various cancer cells and induces apoptotic processes in cancer cells,[24,89] suggesting its inhibitory effects on cancers. It has been found that EGCG had a concurrent effect on two important transcription factors, such as p53 (stabilization of p53) and NF-κB (negative regulation of NF-κB activity), and also caused a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis.[94] Moreover, EGCG has been found to reduce the levels of matrix metalloproteinases, suppress angiogenesis, and inhibit invasion and metastasis.[95,96] In addition, EGCG also prevents oxidative modification of low density lipoproteins in human and the development of atherosclerosis in apoprotein E-deficient mice,[97,98] suggesting that EGCG may reduce the risk of cardiovascular diseases.

4.5. *Resveratrol*

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin present in a wide variety of plant species including grapes, mulberries, and peanuts. Relatively high quantities of resveratrol are found in grapes. The concentration of resveratrol in red wine and grape juice is in the range of 0.05–10 mg/l, depending on grape cultivar, geographical origin, and process methodology.[10] Resveratrol has been shown to have beneficial effects

on the reduction of oxidative stress and the prevention of heart diseases, degenerative diseases, and cancers.[10,99,100]

Resveratrol has been reported to modulate lipoprotein metabolism and to inhibit platelet aggregation and coagulation,[101] suggesting its preventive effects on cardiovascular diseases. It has been found that resveratrol reduces DNA damage and formation of A2E-epoxidation, which is implicated in the degenerative disease.[102] Moderate wine consumption has been associated with decreased odds of developing age-related degenerations.[103] Experimental studies have shown that resveratrol inhibits the growth of various cancer cells and induces apoptotic cell death.[104–107] The induction of apoptosis by resveratrol has been believed to be mediated through p53-depenedent, Fas, MAPK, or ceramide signaling pathway.[104–107] Resveratrol also shows their inhibitory effects on the activity of NF-κB,[18] suggesting its role as antioxidant contributing to cancer prevention and/or treatment.

4.6. *Lycopene*

Tomatoes are rich in lycopene, which is the pigment principally responsible for the deep-red color of tomato and its products. Tomato products including ketchup, tomato juice, and pizza sauce are the richest sources of lycopene in the US diet. The consumption of tomatoes and tomato products containing lycopene have been shown to be associated with decreased risk of chronic diseases such as cardiovascular diseases and cancers.[11]

Lycopene is a potent antioxidant. It has been found that lycopene, as a biologically occurring carotenoid, exhibits high physical quenching rate constant with singlet oxygen, suggesting its high activity as antioxidant.[108] Sesso *et al.*[109] have found that higher plasma lycopene concentrations are associated with a lower risk of cardiovascular diseases in women. The prevention of lipid peroxidation by lycopene may be one of the reasons that lycopene reduces the risk of atherosclerosis and cardiovascular diseases.[110] In addition to the effect on cardiovascular disease, lycopene also shows beneficial effects on cancer prevention and treatment. Giovannucci *et al.*[111] have reported that frequent consumption of tomato products is associated with a lower risk of prostate cancer. The inverse associations between plasma lycopene and prostate cancer have also been reported.[112] Experimental studies also show that lycopene inhibits cell growth in breast, prostate, and

endometrial cancer cells with regulation of cell cycle-related genes.[113,114] Clinical trial have revealed that lycopene supplements reduce tumor size and PSA level in localized prostate cancers,[115] suggesting its promising effects on prostate cancer treatment.

4.7. *Vitamins and others*

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant distributed in green leaf vegetables, nuts, seeds, sunflower, and plant oils. Plant oils are the main dietary source of vitamin E. Vitamin E exerts potent antioxidant effect. It has been reported that vitamin E inhibits NF-κB activation and NF-κB-dependent transcription, and induces differentiation through reduction of NF-κB,[19,116,117] suggesting that vitamin E may exert its antioxidant effect through modulation of NF-κB. Vitamin E supplement has been associated with decreased risk of degenerative disease and cardiovascular disease.[118,119] Vitamin E also shows its inhibitory effects on carcinogenesis.[120] Vitamin E and its deriver have been known to inhibit cancer cell growth via modulating cell cycle regulatory and apoptotic machineries,[121,122] suggesting their inhibitory effects on cancers.

Vitamin C is a water-soluble antioxidant. The sources of vitamin C are fruits and vegetables, particularly orange, strawberry, citrus, kiwi, Brussels sprouts, and cauliflower.[5] It has been reported that vitamin C inhibits NF-κB activation by the inhibition of IκBα phosphorylation or the activation of p38 mitogen-activated protein kinase.[20,123]

In addition to vitamin E and C, vitamin A, ginseng, ubiquinone, ginkgo, and docosahexaenoic acid have also been known as antioxidants.[22] They may have some beneficial effects on human health, particularly in chronic diseases including cancers.

5. Conclusions

Oxidative stress has been linked to aging, some chronic diseases, and carcinogenesis. NF-κB plays important roles in oxidative stress and carcinogenesis. Therefore, targeting NF-κB may be a novel and important preventive or therapeutic strategy against some chronic diseases and

cancers. The plant-derived components (isoflavones, curcumin, EGCG, I3C, resveratrol, lycopene, vitamin E, vitamin C, etc.) have been found to reduce oxidative stress and inhibit NF-κB activation. They may have inhibitory effects on atherosclerosis, neurodegeneration, oncogenesis, cancer cell growth, and progression. These effects make them strong candidates as chemopreventive or therapeutic agents against cardiovascular diseases, degenerative diseases, and cancers.

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Research Article

Inhibition of Angiogenesis and Invasion by 3,3′-Diindolylmethane Is Mediated by the Nuclear Factor–κB Downstream of Target Genes *MMP-9* and *uPA* that Regulated Bioavailability of Vascular Endothelial Growth Factor in Prostate Cancer

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Abstract

Progression of prostate cancer is believed to be dependent on angiogenesis induced by tumor cells. 3,3′-Diindolylmethane (DIM) has been shown to repress neovascularization in a Matrigel plug assay and inhibit cell proliferation, migration, invasion, and capillary tube formation of cultured human umbilical vein endothelial cells. However, the molecular mechanism, by which DIM inhibits angiogenesis and invasion, has not been fully elucidated. Therefore, we sought to explore the molecular mechanism by which DIM inhibits angiogenesis and invasion, specifically by investigating the role of angiogenic factors secreted by prostate cancer cells which control all steps of angiogenesis. We found that BioResponse DIM (B-DIM), a formulated DIM with higher bioavailability, inhibited angiogenesis and invasion by reducing the bioavailability of vascular endothelial growth factor (VEGF) via repressing extracellular matrix–degrading proteases, such as matrix metalloproteinase (MMP)-9 and urokinase-type plasminogen activator (uPA), in human prostate cancer cells and reduced vascularity (angiogenesis) *in vivo* using Matrigel plug assay. We also found that B-DIM treatment inhibited DNA binding activity of nuclear factor-κB (NF-κB) which is known to mediate the expression of many NF-κB downstream target genes, including VEGF, IL-8, uPA, and MMP-9, all of which are involved in angiogenesis, invasion, and metastasis. Our data suggest that inhibition of NF-κB DNA binding activity by B-DIM contributes to the regulated bioavailability of VEGF in MMP-9 and uPA and, in turn, inhibits invasion and angiogenesis, which could be mechanistically linked with the antitumor activity of B-DIM as observed previously by our laboratory in a prostate cancer animal model. [Cancer Res 2007;67(7):1–10]

Introduction

The growth and metastasis of prostate cancer and other tumors is dependent on the induction of new blood vessels from preexisting ones through angiogenesis (1). Growing evidence shows that prostate cancer cells secrete high levels of growth factors and matrix-degrading proteases, including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor, matrix metalloproteinases (MMP; ref. 2), and serine proteases, in particular, the urokinase-type plasminogen activator (uPA; ref. 3). Many studies have documented that VEGF is a critical mediator of angiogenesis and regulates most of the steps in the angiogenic cascade, including proliferation, migration, and tube formation of endothelial cells (4, 5). VEGF exists in at least six isoforms with variable amino acid residues produced through alternative splicing: VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206 (6). VEGF121, VEGF165, and VEGF189 are the major forms secreted by most cell types. After secretion, VEGF121 diffuses relatively freely in tissues, whereas approximately half of the secreted VEGF165 binds to heparin sulfate proteoglycans (HSPG) on the cell surface and matrix basement membrane. VEGF189 remains almost completely sequestered by HSPGs in the extracellular matrix (ECM), making the HSPGs a reservoir of VEGF that can be released by proteolysis (6). VEGF is subjected to multilevel regulation to ensure proper expression under physiologic and pathologic conditions. A wide range of cytokines, oncogenic proteins, or transcription factors, such as insulin-like growth factor 1, c-Src, Ras, nuclear factor-κB (NF-κB), and SP1, have been shown to regulate VEGF gene transcription (7–9). Hypoxic condition can also affect VEGF mRNA stability (10) and translation initiation (11). Extracellular cleavage of VEGF by uPA (12, 13) or plasmin (14) and processing by MMPs can regulate its mitogenic effect, bioavailability, and vascular patterning in tumors (15). These multilevel regulated patterns of VEGF provide various targets for the inhibition of angiogenesis induced by tumors.

Epidemiologic studies have shown a strong inverse correlation between consumption of cruciferous vegetables and a risk of prostate cancer (16). One of the bioactive components of these vegetables is indole-3-carbinol. Indole-3-carbinol, in a low-pH environment, is easily converted to its dimeric product 3,3′-diindolylmethane (DIM). DIM can inhibit the growth of tumors through induction of apoptosis and G_1 cell-cycle arrest in human prostate cancer cells (17, 18). Recent studies have shown that DIM inhibits cell proliferation, migration, invasion, and capillary tube formation of cultured human umbilical vascular endothelial cell (HUVEC) and represses vascularization in a Matrigel plug assay and tumorigenesis in human breast cancer cell xenograft model in mice (19). Another study showed that DIM inhibits angiogenesis via inhibition of growth factor–induced Ras signaling in HUVECs (20). However, the molecular mechanism by which DIM inhibits angiogenesis and invasion has not been fully elucidated. Tumor angiogenesis involves degradation of the basement membrane of the original vessel, endothelial cell activation, migration, proliferation, and formation of new capillaries. All these processes are controlled by angiogenic factors secreted either by the tumor or the surrounding stroma. During the process of tumor angiogenesis, tumor cells express high levels of angiogenic factors, such as VEGF, MMP-9, and uPA, which are important for angiogenesis,

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invasion, and metastasis. Little is known about whether DIM is able to inhibit angiogenesis in prostate tumor cells by regulating these angiogenic factors. In this report, we show that BioResponse DIM (B-DIM), a formulated DIM with higher bioavailability, inhibits angiogenesis and invasion by reducing the bioavailability of VEGF due to decreased expression of uPA and MMP-9, resulting from the inhibition of NF-κB DNA binding activity in human prostate cancer cells.

Materials and Methods

Cell lines and culture. Prostate cancer cell lines LNCaP (AR^+ and responsive to androgen) and C4-2B (AR^+ but nonresponsive to androgen) cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 10 μmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin. HUVECs were purchased from American Type Culture Collection (Manassas, VA) and cultured in F12K medium (American Type Culture Collection) supplemented with 10% FBS, 0.1 mg/mL heparin sulfate, 0.05 mg/mL endothelial cell growth factor supplement (BD Bioscience, San Jose, CA), 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured in a 5% CO_2–humidified atmosphere at 37°C.

Reagent and antibody. B-DIM, a formulated DIM with higher bioavailability, was kindly provided by Dr. Michael Zeligs (BioResponse, Boulder, CO) and was dissolved in DMSO to make 50 mmol/L stock solutions and stored at −20°C in multiple aliquots. Antibody against human VEGF (SC-152, A-20) and anti-uPA antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti–MMP-9 monoclonal antibody was obtained from R&D Systems (Minneapolis, MN). The monoclonal antibody to β-actin was purchased from Sigma-Aldrich (St. Louis, MO). Matrigel, growth factor–reduced Matrigel, and recombinant human VEGF165 were obtained from BD Bioscience (Bedford, MA). VEGF neutralizing antibody was obtained from Lab Vision (Fremont, CA). Anti-CD31 antibody was purchased from Ventana Medical Systems, Inc. (Tucson, AZ). VEGF quantikine ELISA kits and MMP-9 fluorescent assay kits were purchased from R&D Systems.

Matrigel *in vitro* HUVECs tube formation assay. LNCaP and C4-2B cells cultured in serum-free RPMI 1640 were treated with B-DIM for 24 h. The conditioned media were collected, centrifuged, transferred to fresh tubes, and stored at −20°C. Growth factor–reduced Matrigel (125 μL), after being thawed on ice, was plated in an 8-well chamber (Fisher). The chamber was then incubated at 37°C for 30 min to allow the Matrigel to polymerize. HUVECs maintained in F12K complete medium and starved for 4 h were trypsinized and seeded (5×10^4 cells per well) in each well with 250 μL of conditioned medium from LNCaP or C4-2B cells treated with DMSO or 10 μmol/L of B-DIM in serum-free RPMI 1640 for 24 h. VEGF165 (50 ng/mL) was used as a positive control and medium only was used as a negative control. Before being added into the HUVECs, conditioned media from LNCaP and C4-2B cells were preincubated with anti-VEGF neutralizing antibody at 37°C for 1 h to confirm that the tube formation induced by conditioned medium is related to VEGF secreted by prostate cancer cells. HUVECs grown in growth factor–reduced Matrigel were treated with DMSO or 10 μmol/L B-DIM for 6 h to observe whether B-DIM directly affects tube formation of HUVECs, The chamber was incubated for 6 h. Each well was photographed using an inverted microscope with digital camera. Image analysis of tubule/capillary length was carried out using the software image analysis program Scion Image downloaded from the NIH website.[1]

ELISA assay for VEGF and MMP-9. LNCaP and C4-2B cells were seeded in six-well plates. After 24 h, the cells were incubated with serum-free medium for another 24 h and then treated with B-DIM or DMSO as a vehicle control in 1% FBS for 24 h. The culture media were collected, centrifuged to remove cellular debris, and stored at −70°C until assay for VEGF and MMP-9. In addition, LNCaP and C4-2B cells were seeded in six-well plates. After 24 h, the cells were transfected with MMP-9 plasmid or pcDNA3 control plasmid using effectene transfection reagent (Qiagen, Valencia, CA). The media were removed after 18 h of transfection, and the cells were treated with 10 μmol/L B-DIM in serum-free media and incubated for 24 h. To investigate the effects of uPA on the release of VEGF, LNCaP and C4-2B cells were transfected with uPA small interfering RNA (siRNA; Santa Cruz) or control siRNA using DharmaFECT3 siRNA transfection reagent (Dharmacon, Lafayette, CO). The media were removed after 18 h of transfection, and the cells were incubated in serum-free media for 24 h. The culture media were collected, centrifuged to remove cellular debris, and stored at −70°C until assay for VEGF. The cells in the plate were trypsinizied, and the total number of cells was determined by cell counting. The assay was done using a commercially available VEGF and MMP-9 ELISA kit (R&D System) according to the manufacturer's instruction. For MMP-9 assay, the culture media were concentrated using the microcon concentrator (Millipore). Results were normalized to the cell number.

Real-time reverse transcription–PCR. LNCaP and C4-2B cells grown in 1% FBS were treated with 10 μmol/L B-DIM or DMSO as a vehicle control for 24 h. The total RNA was isolated using the Trizol reagent. One microgram of RNA was reverse transcribed using a reverse transcription system (Invitrogen) according to the manufacturer's instruction. Real-time PCR was used to quantify mRNA expression. Sequence of oligonucleotide primers and probes for total VEGF, VEGF121, VEGF165, and VEGF189 used in this study was described by Dr. Gustafsson (21). β-Actin was used for internal control to correct the potential variation in RNA loading. Targets and β-actin gene were run under the same conditions. All reactions were done in a 25-μL volume containing the sample cDNA, TaqMan fast universal PCR mastermix, primers, and probes. Before the PCR cycles, samples were incubated for 2 min at 50°C and 10 min at 95°C. Thermal cycles consisted of 45 cycles at 95°C for 15 s and 65°C for 1 min.

Western blot assay. LNCaP and C4-2B cells were treated as above. The cells were harvested by scraping from the wells and washed twice with cold PBS. The cell pellet was suspended in 125 mmol/L Tris-HCl (pH 6.8), sonicated for 10 s, and an equal volume of 4% SDS was added. The lysates were boiled for 10 min. Protein concentration was determined using bicinchoninic acid protein assay (Pierce, Rockford, IL). The proteins were separated by SDS-PAGE, transferred to nitrocellulose, blocked, and incubated with the following primary antibodies: VEGF (1:1,000, Santa Cruz), MMP-9 (2 μg/mL, R&D Systems), uPA (1:500, Santa Cruz), and β-actin (1:10,000, Sigma). The membrane was washed and incubated with the respective secondary antibodies conjugated with peroxidase. Protein detection was done with chemiluminescence detection system (Pierce).

Electrophoretic mobility shift assay for NF-κB DNA binding activity. LNCaP and C4-2B cells were seeded in a six-well plate. After 24 h, the cells were treated with 10 μmol/L of B-DIM or DMSO as a vehicle control and incubated for another 24 h. Nuclear extracts were prepared according to the method described by Wang et al. (22). For detecting NF-κB DNA binding activity, electrophoretic mobility shift assay (EMSA) was done by incubating 3 μg of nuclear protein of each sample with IRDye 700–labeled NF-κB oligonucleotide (LI-COR, Lincoln, NE) as described (22). Retinoblastoma protein was used as a protein loading control.

Invasion assay. The effects of B-DIM treatment on cell invasion were determined using BD BioCoat Tumor Invasion Assay System (BD Bioscience, Bedford, MA) according to the instruction of the manufacturer. Briefly, LNCaP and C4-2B cells with serum-free media containing 10 and 25 μmol/L of B-DIM or DMSO were seeded into the upper chamber of the system. Bottom wells were filled with complete media. After 24 h incubation, the cells in the upper chamber were removed and the cells invaded through the Matrigel membrane were stained with 4 μg/mL Calcein AM in PBS at 37°C for 1 h. The fluorescently labeled cells were photographed under a fluorescence microscope. The fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm.

Transient transfections and reporter gene assay. The plasmid pGL3-V2274 containing the VEGF promoter, enhancer, and firefly luciferase reporter gene was kindly provided by Dr. Keping Xie (University of Texas M.D. Anderson Cancer Center). LNCaP and C4-2B cells were seeded at a

[1] http://www.scioncorp.com

density of 8×10^3 per well in 96-well plate and incubated for 24 h. The cells were cotransfected with pGL3-V2274 or pGL3-basic plasmid and cytomegalovirus (CMV)-β-galactosidase plasmid. After 24 h of transfection, the cells were treated with 10 μmol/L of B-DIM or DMSO as vehicle control for another 24 h. Luciferase activity was assayed using Steady-Glo Luciferase Assay System (Promega). β-Galactosidase activity was used as a control for transfection efficiency. Q8

***In vivo* Matrigel plug angiogenesis assay.** Our data showed that the condition medium from LNCaP and C4-2B cells treated with B-DIM reduced the tube formation of HUVECs *in vitro* through reduction in bioavailability of VEGF. To assess whether B-DIM inhibits angiogenesis *in vivo*, angiogenesis assay was done using male CB17 SCID mice. The mice were randomly segregated into two groups (four mice per group). C4-2B cells (4×10^6 in 50 μL PBS admixed with 0.25 mL Matrigel) were injected s.c. into the bilateral flanks of each mouse (eight lesions per group). The control group of mice was gavaged with the vehicle (sesame oil), whereas the other group of mice was gavaged with B-DIM (5 mg/kg body weight, daily) for 4 weeks. On termination, Matrigel plugs with tumor cells were harvested and fixed in 10% formalin dehydrated and embedded in paraffin. Matrigel plug slices were stained with H&E and anti-CD31 antibody. SPOT Advanced Imaging software was used to capture bright-field images. Image analyses of the area of CD31-positive blood vessels, vessel number, and length of vessel perimeter in each of the entire field were carried out using software image analysis program (Scion Image downloaded from NIH website).[1]

Data analysis. Experiments presented in the figures are representative of three or more different repetitions. The data are presented as the mean

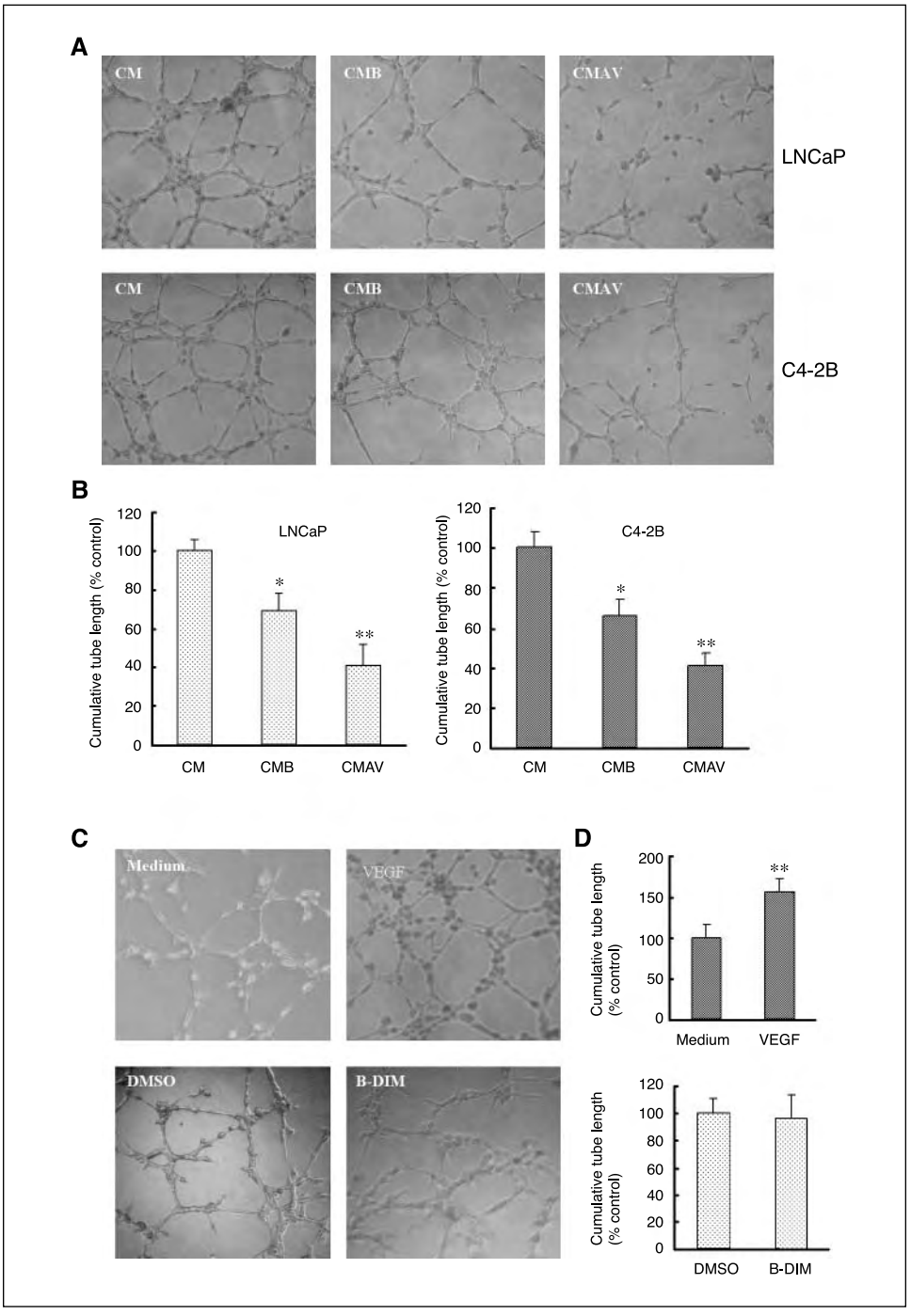

Figure 1. Reduced tube formation of HUVECs induced by conditioned media from LNCaP and C4-2B cells treated with B-DIM. Growth factor–reduced Matrigel (125 μL) after being thawed on ice was plated in an eight-well chamber. The chamber was then incubated at 37°C for 30 min to allow the Matrigel to polymerize. HUVECs (5×10^4 cells per well) were seeded into each well in conditioned medium. *A,* conditioned medium was from LNCaP and C4-2B cells treated with either DMSO (*CM*) or treated with 10 μmol/L B-DIM (*CMB*) in serum-free medium for 24 h and conditioned medium from LNCaP and C4-2B cells preincubated with 10 μg/mL VEGF neutralizing antibody at 37°C for 1 h (*CMAV*). The chamber was incubated for 6 h. The well was photographed using an inverted microscope with a digital camera. Results are representative of three independent experiments. *B,* image analysis of tubule/capillary length was carried out using software image analysis program Scion Image downloaded from NIH website. Quantification of cumulative tube length of endothelial cells as observed in (*A*). *Columns,* mean; *bars,* SE. $n = 4$. *, $P < 0.05$; **, $P < 0.01$ compared with conditioned media control. *C,* medium only was used as negative control; 50 ng/mL VEGF was used as positive control. To rule out the possibility that inhibition of tube formation by conditioned media from LNCaP and C4-2B cells treated with 10 μmol/L of B-DIM was mediated through direct effect of B-DIM on tube formation of HUVECs within 6 h incubation, HUVECs were cultured in RPMI 1640 containing DMSO or 10 μmol/L of B-DIM. The chamber was incubated for 6 h. The well was photographed using an inverted microscope with a digital camera. Results are representative of three independent experiments. *D,* image analysis of tubule/capillary length was carried out using software image analysis program Scion Image downloaded from NIH website. Quantification of cumulative tube length of endothelial cells cultured in medium alone or 50 ng/mL VEGF (*top*) and treated with DMSO or 10 μmol/L B-DIM (*bottom*). *Columns,* mean; *bars,* SE. $n = 4$. **, $P < 0.01$ compared with medium alone.

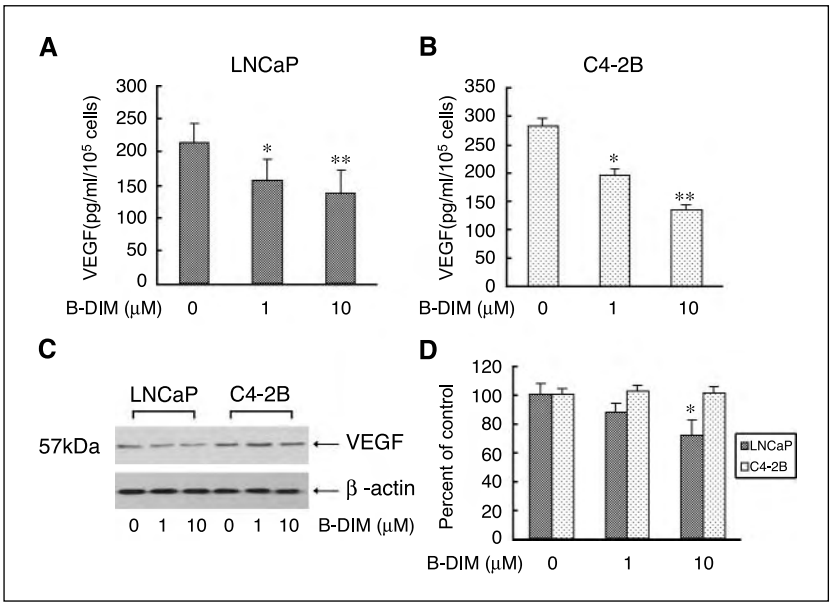

Figure 2. The effects of B-DIM on the secretion and expression of cellular VEGF. LNCaP (*A*) and C4-2B (*B*) cells were seeded at a density of 2×10^5 per well in a six-well plate. After 24 h, the cells were incubated with serum-free medium for another 24 h. The cells were then treated with 1 and 10 μmol/L of B-DIM or DMSO as vehicle control in 1% FBS for 24 h. The culture media was collected, and VEGF was assayed using an ELISA kit. Results were normalized to the cell number and expressed as pg/mL/10^5 cells. *Columns,* mean; *bars,* SE. $n = 6$. *, $P < 0.05$; **, $P < 0.01$ compared with control. *C,* cell culture conditions same as above, the cell lysates were subjected to gel electrophoresis and Western blot analysis was done using anti-VEGF antibody. β-Actin protein was used as loading control. *D,* quantitative analysis of VEGF expression in LNCaP and C4-2B cells was done and relative density of bands normalized for β-actin (control was assigned the value of 100%). *Columns,* mean; *bars,* SE. $n = 3$. *, $P < 0.05$ compared with control.

values ± SE. Comparisons between groups were evaluated by a two-tailed Student's *t* test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Reduced tube formation of HUVECs induced by conditioned media from LNCaP and C4-2B cells treated with B-DIM. Tumor cells can induce the formation of new blood vessels by secreting endothelial cell specific growth factors including VEGF and FGF. To determine whether conditioned media from LNCaP and C4-2B cells could induce the tube formation of HUVECs and whether conditioned media from LNCaP and C4-2B cells treated with B-DIM could reduce the tube formation, we did the tube formation assay in growth factor–reduced Matrigel *in vitro*. Conditioned media from LNCaP and C4-2B cells (Fig. 1*A* and *B*) as well as VEGF (Fig. 1*C*) were able to significantly induce the tube formation of HUVECs in 6-h incubation (compared with Fig. 1*C*, medium only as negative control). To further explore whether VEGF in conditioned media may be the inducer of the tube formation of HUVECs, 10 μg/mL of anti-VEGF neutralizing antibody was preincubated with conditioned media from LNCaP and C4-2B cells at 37°C for 1 h and then added into HUVECs. We found that anti-VEGF neutralizing antibody reduced the tube formation, suggesting that VEGF is the inducer of the tube formation induced by conditioned medium from LNCaP and C4-2B cells (Fig. 1*A*). Importantly, we found that conditioned media from LNCaP and C4-2B cells treated with 10 μmol/L of B-DIM inhibited tube formation (Fig. 1*A*). To rule out the possibility that inhibition of tube formation by conditioned media from LNCaP and C4-2B cells treated with 10 μmol/L of B-DIM was mediated through direct effect of B-DIM on tube formation of HUVECs, HUVECs grown in growth factor–reduced Matrigel were treated with 10 μmol/L of B-DIM for 6 h (Fig. 1*C*). B-DIM had no effect on tube formation within 6 h of incubation (compared with Fig. 1*C*, DMSO control). Figure 1*B* and *D* showed quantification of cumulative tube length of endothelial cells. These results suggest that B-DIM treatment inhibits tube formation by inhibiting secretion of proangiogenic factor VEGF in LNCaP and C4-2B cells.

B-DIM reduced cellular VEGF and the secretion of VEGF. To directly determine whether the reduced tube formation seen earlier was a consequence of the decreased secretion of VEGF, we analyzed the levels of VEGF in conditioned media from LNCaP and C4-2B cells treated with B-DIM. As shown in Fig. 2*A* and *B*, 1 and 10 μmol/L of B-DIM treatment markedly decreased the VEGF secretion from LNCaP and C4-2B cells compared with DMSO control ($P < 0.05$ or 0.01). Western blot analysis showed that B-DIM treatment reduced cellular VEGF in LNCaP cells but had no significant effect on cellular VEGF in C4-2B cells (Fig. 2*C* and *D*). The band with 57 kDa molecular weight corresponded with the long intact isoforms of VEGF189 predominantly associated with cell surface and ECM. The long VEGF189 may be the storage form, which is released and activated by two serine proteases, uPA and plasmin (13).

B-DIM down-regulated VEGF at the level of transcription in LNCaP but not in C4-2B cells. Houck et al. reported that VEGF bioavailability could be dually regulated at the genetic level by alternative splicing of VEGF mRNA that determines whether VEGF will be soluble or incorporated into a biological reservoir and later activated through proteolysis after plasminogen activation (14). VEGF121, VEGF165, and VEGF189 are the major forms secreted by most cell types. To determine whether the decreased secretion of VEGF from LNCaP and C4-2B cells treated with B-DIM was due to transcription down-regulation of soluble isoforms of VEGF, such as VEGF121 and VEGF165, we conducted real-time reverse transcription–PCR (RT-PCR) assay using isoform-specific VEGF primers and probes and tested the relative distribution of mRNA levels of major isoforms in LNCaP and C4-2B cells, respectively. We found that VEGF121 is the most abundant in prostate cancer cell line LNCaP and C4-2B cells; and VEGF165 is the lowest (Fig. 3*A*). Importantly, we found that total VEGF and VEGF121 was significantly reduced by 10 μmol/L of B-DIM treatment, but VEGF165 and VEGF189 showed less reduction at the RNA level in LNCaP cells. However, 10 μmol/L of B-DIM treatment had no significant effect on all isoforms of VEGF mRNA in C4-2B cells (Fig. 3*B*). To further test whether B-DIM reduced the VEGF expression at the transcriptional level, we used a reporter construct, pGL3-V2274 (VEGF 5′ flanking

region from +50 to −2274), which contains a luciferase gene driven by the VEGF promoter. This reporter plasmid was transiently transfected into LNCaP and C4-2B cells. After 24 h of transfection, the cells were treated with 10 μmol/L of B-DIM for another 24 h. The luciferase activity was determined. B-DIM treatment induced significant decrease in luciferase activity of LNCaP but not C4-2B cells (Fig. 3*C*). Therefore, we speculated that decreased soluble VEGF in culture media from C4-2B cells treated with B-DIM may result from regulation of posttranscription or the reduced bioavailability of bound-VEGF, such as VEGF189 and VEGF165. Many studies indicated that serine protease and matrix-degrading proteases were able to release bound-VEGF into tissue culture media (13–15). uPA and MMP-9 are respectively the key serine protease and matrix-degrading protease involved in the regulation of bioavailability of VEGF; thus, we have investigated whether expression of MMP-9 could regulate the bioavailability of VEGF.

B-DIM reduced bioavailability of VEGF through repressing MMP-9 expression. MMP-9 has been shown to be expressed at higher levels in prostate cancer cell lines and prostate cancer tissue. It is known to specially cleave the type IV collagen, the major ECM, and is able to regulate bioavailability of ECM-binding VEGF via degrading the ECM (2, 23). In this study, we determined MMP-9 levels from conditioned media. As shown in Fig. 4*A* and *B*, LNCaP and C4-2B cells treated with 1, 10, and 25 μmol/L of B-DIM for 24 h resulted in significant decrease in the MMP-9 levels in culture supernatants compared with the control (treated with DMSO). In addition, cellular MMP-9 levels in the treated cells were examined by Western blot analysis. As depicted in Fig. 4*A* and *B* (*bottom*), B-DIM dramatically repressed the MMP-9 expression. To explore whether reduced MMP-9 expression by B-DIM was responsible for the decreased bioavailability of VEGF, we assayed the VEGF levels in conditioned media from LNCaP and C4-2B cells transfected with MMP-9 plasmid and treated with B-DIM. The results showed that MMP-9 transfection significantly increases VEGF levels from conditioned media compared with transfection of control plasmid in LNCaP and C4-2B cells. Importantly, 10 μmol/L of B-DIM treatment for LNCaP and C4-2B cells transfected with MMP-9 plasmid dramatically reduced the release of VEGF (Fig. 4*C* and *D*). Western blot analysis showed that B-DIM repressed MMP-9 expression in LNCaP and C4-2B cells transfected with MMP-9 plasmid. These results revealed that B-DIM could reduce bioavailability of VEGF through repressing MMP-9 expression.

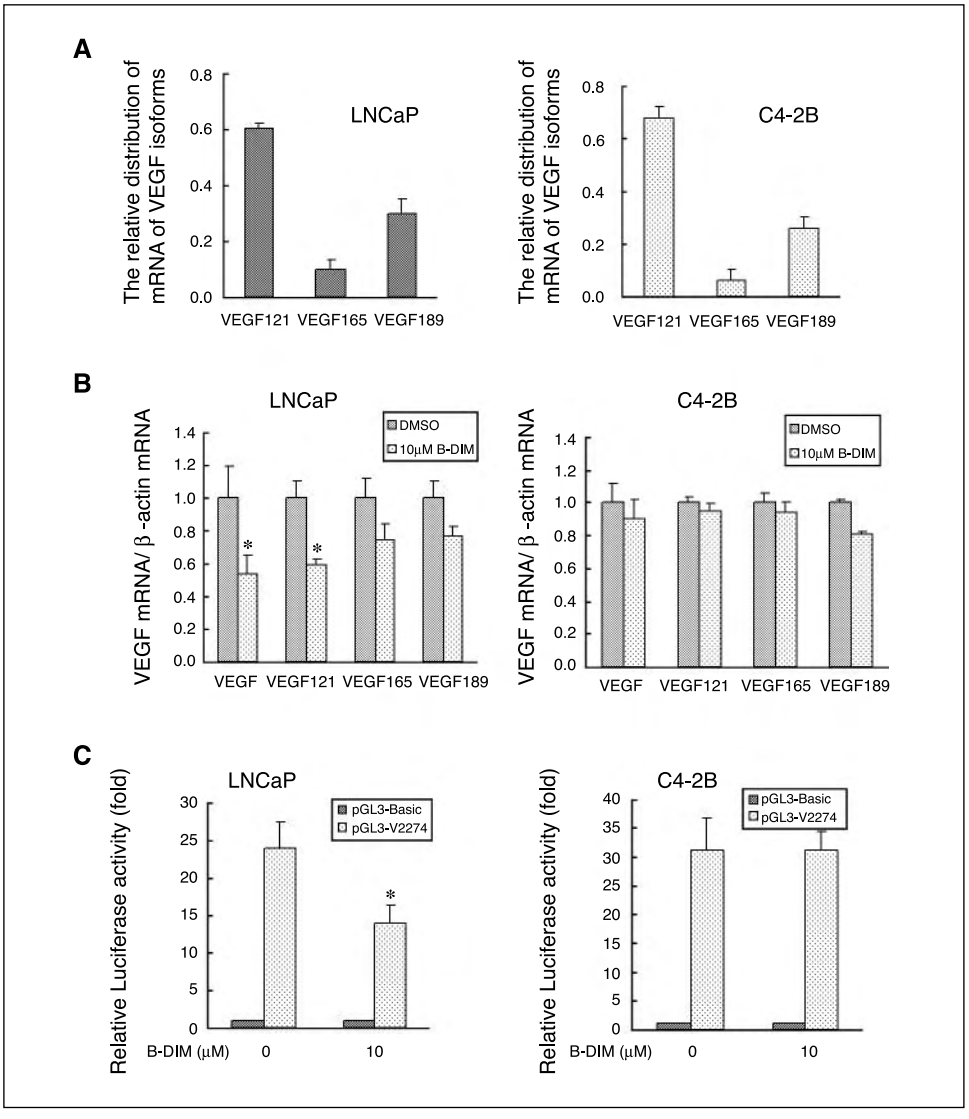

Figure 3. The effects of B-DIM on VEGF mRNA and promoter activity and the relative distribution of mRNA levels of VEGF isoforms. *A,* the relative distribution of mRNA levels of VEGF isoforms in LNCaP and C4-2B cells, respectively. *B,* LNCaP and C4-2B cells grown in 1% FBS were treated with 10 μmol/L of B-DIM or DMSO as control for 24 h. The total RNA was isolated using the Trizol reagent. Real-time RT-PCR was done, and specific primers and probes for total VEGF, VEGF121, VEGF165, and VEGF189 were used to quantify mRNA levels. β-Actin was used for internal control to correct the potential variation in RNA loading. *, $P < 0.05$. $n = 4$. *C,* pGL3-basic luciferase reporter or pGL3-2274 containing the VEGF promoter, enhancer, and firefly luciferase reporter gene were cotransfected with CMV-β-galactosidase plasmid into LNCaP and C4-2B cells. After 24 h of transfection, the cells were treated with 10 μmol/L of B-DIM or DMSO as vehicle control for another 24 h. Luciferase activity was determined using Steady-Glo Luciferase assay system (Promega). β-Galactosidase activity was used as a control for transfection efficiency. Fold increase in luciferase activity was calculated relative to the luciferase activity of pGL3-basic assigned the value of 1. B-DIM (10 μmol/L) treatment decreased the VEGF promoter activity in LNCaP but not in C4-2B cells. *, $P < 0.05$ compared with without B-DIM control. $n = 6$.

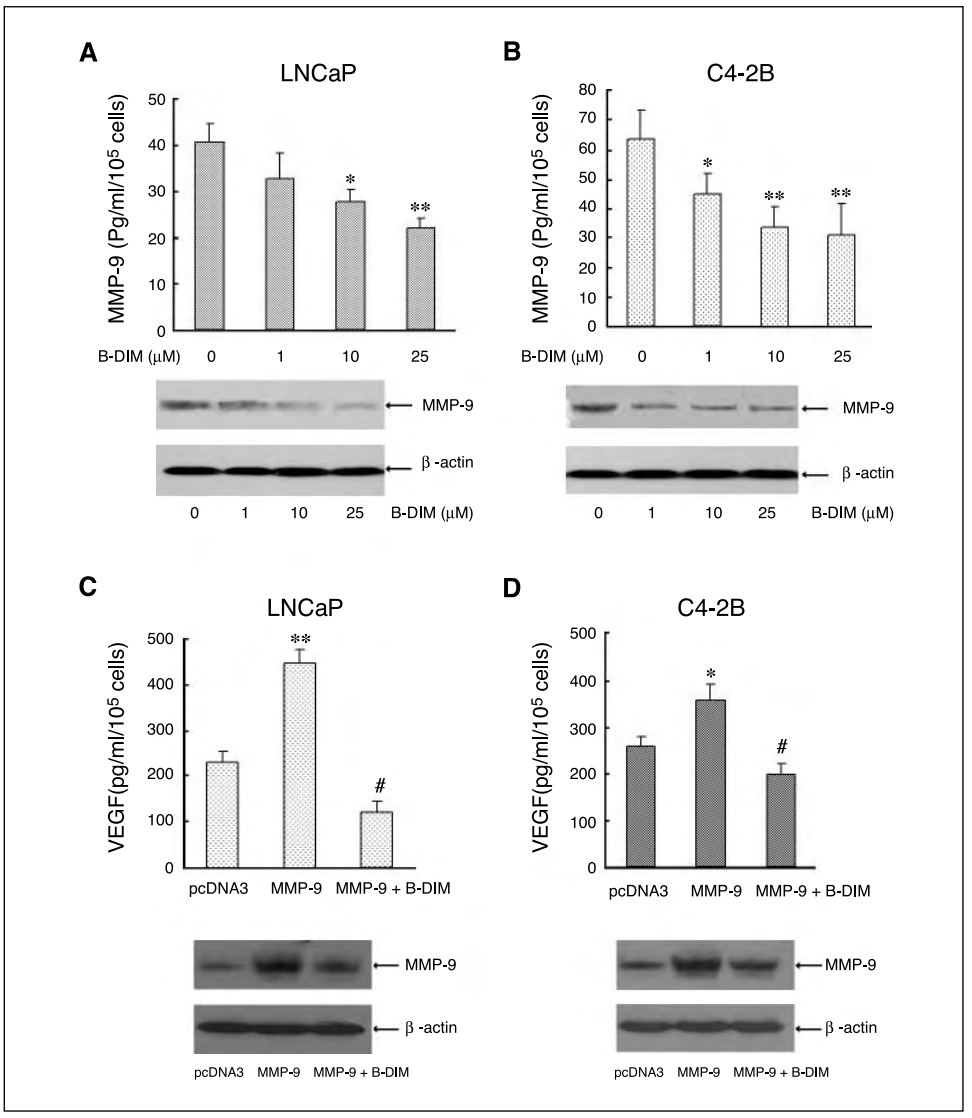

Figure 4. B-DIM repressed secretion and expression of MMP-9 and regulation of VEGF release by MMP-9. LNCaP (*A*)and C4-2B (*B*) cells were seeded at a density of 2×10^5 per well in six-well plate. After 24 h, the cells were cultured in serum-free medium and incubated for another 24 h, and then treated with B-DIM or DMSO as vehicle control in 1% FBS for 24 h. The culture media were collected and concentrated using the Microcon concentrator. MMP-9 levels were assayed using ELISA kit. The results were normalized to the cell number. The cell lysates were subjected to Western blotting using anti–MMP-9 antibody. β-Actin protein was used as loading control. *, $P < 0.05$; **, $P < 0.01$ compared with without B-DIM control. LNCaP (*C*) and C4-2B (*D*) cells were transfected with MMP-9 plasmid or pcDNA3 control plasmid using the effectene transfection reagent. After 18 h transfection, cells were treated with 10 μmol/L of B-DIM in serum-free media and incubated for 24 h. The culture media were collected for VEGF assay. Cell lysates were subjected to Western blotting using anti–MMP-9 antibody. β-Actin protein was used as loading control. *, $P < 0.05$; **, $P < 0.01$ compared with pcDNA control; #, $P < 0.01$ compared with MMP-9 group.

Because uPA also plays an important role in the regulation of VEGF bioavailability, we next examined the effect of B-DIM on uPA and its consequence in VEGF bioavailability.

uPA regulated the bioavailability of the VEGF and B-DIM repressed uPA expression in LNCaP and C4-2B cells. Many studies have shown that uPA is involved in the angiogenesis induced by tumors (24). uPA, directly or via plasmin formation, leads to the release or activation of many angiogenic growth factors, such as basic FGF (bFGF) and VEGF. In this study, down-regulation of uPA by siRNA significantly repressed the release of VEGF in LNCaP and C4-2B cells (Fig. 5*A*). This result suggests that B-DIM may decrease the bioavailability of VEGF via repressing the uPA expression. In this study, we found that 10 μmol/L of B-DIM treatment significantly decreased the uPA expression in LNCaP and C4-2B cells compared with DMSO control (Fig. 5*B*). These results suggest that B-DIM may regulate the bioavailability of bound-VEGF by suppressing MMP-9 and uPA expression to inhibit angiogenesis induced by prostate cancer cells. It is known that VEGF, uPA, and MMP-9 are transcriptionally regulated by NF-κB; thus, we have tested whether B-DIM regulated expression of VEGF, uPA, and MMP-9 is mediated through NF-κB.

B-DIM inhibited the NF-κB DNA binding activity. NF-κB activation mediates expression of a large number of target genes, such as VEGF, MMP-9, and uPA, which are involved in angiogenesis and invasion (25). To further explore the mechanism by which B-DIM regulates the expression of VEGF, MMP-9, and uPA, we detected NF-κB DNA binding activity. Nuclear extract from cells treated with B-DIM or DMSO control were subjected to EMSA. Figure 5*C* showed that treatment with 10 μmol/L of B-DIM for 24 h strongly inhibited NF-κB DNA binding activity in both LNCaP and C4-2B cells, suggesting that down-regulation of VEGF, MMP-9, and uPA by B-DIM could be partly due to the inhibition of NF-κB DNA binding activity, especially in LNCaP cells. However, the regulatory mechanism of VEGF, MMP-9, and uPA by B-DIM in C4-2B cells could be more complex compared with those in LNCaP cells.

B-DIM inhibited LNCaP and C4-2B cell invasion. The levels of VEGF, MMP-9, and uPA are known to correlate with prostate cancer invasion and metastasis. Our previous report showed that down-regulation of VEGF and MMP-9 by siRNA inhibited the invasion of human pancreatic cancer cells (22). In this study, we found that B-DIM treatment was able to lower the bioavailability of

VEGF by inhibiting MMP-9 and uPA expression and NF-κB DNA binding activity. We have speculated that B-DIM may inhibit cell invasion of LNCaP and C4-2B due to inactivation of NF-κB, MMP-9, and uPA. Therefore, we did the Matrigel invasion assay. The results showed that 10 and 25 μmol/L of B-DIM significantly inhibited LNCaP (Fig. 6*A*) and C4-2B (Fig. 6*B*) cell invasion. These results suggest that B-DIM inhibits LNCaP and C4-2B cell invasion by decreasing expression or bioavailability of VEGF via inhibiting matrix-degrading proteases, such as uPA and MMP-9.

B-DIM inhibits *in vivo* angiogenesis. The effect of B-DIM on angiogenesis was investigated *in vivo* using a s.c. implanted Matrigel plug assay. Histologic analysis showed that a larger number of endothelial cells were well organized into capillary or large vascular cavities engorged with RBC in the control group, whereas B-DIM treatment strongly inhibited the formation of large vessels (Fig. 6*C*, *middle* and *right* show histologic pictures of peripheral and deeper areas of Matrigel plugs, respectively). In addition, B-DIM treatment also significantly reduced the vessel number (Fig. 6*D*, *left*), vascular area (*middle*) and total length of vascular perimeter (*right*) relative to the control group. These results provide strong evidence in support of the antiangiogenic activity of B-DIM *in vivo*, which is believed to be partially responsible for the antitumor activity of B-DIM as observed previously by our laboratory in a prostate cancer animal model (26).

Discussion

Angiogenesis is a tightly regulated event critical for tumor growth beyond 2 to 3 mm in diameter and is an essential component of tumor metastasis (1). Tumor angiogenesis involves degradation of the basement membrane of the original vessel, endothelial cell activation, migration, proliferation and the formation of new capillaries. All of these processes are controlled by angiogenic factors secreted either by the tumor or the surrounding stroma. VEGF, known as a potential angiogenic factor, is coded by a single gene and coding region that contains eight exons separated by seven introns (27). VEGF exists in at least six isoforms produced through alternative splicing (6). VEGF121, VEGF165, and VEGF189 are the major forms secreted by most cell types. Exons 1, 2, 3, 4, 5, and 8 are common to all isoforms. In addition, VEGF165 contains exon 7 and VEGF189 contains exons 6 and 7. Exons 6 and 7 have been shown to encode the ECM-binding domain of the VEGF. This domain is able to bind HSPGs and other matrix proteins (6). Thus, after secretion, VEGF189 and part of VEGF165 bind to ECM and cell membrane. Extracellular enzymes, such as heparinases, plasmin, uPA, and MMP are able to release bound-VEGF and regulate the VEGF bioavailability.

In the present study, we showed that conditioned media from LNCaP and C4-2B cells induced tube formation of HUVECs. Conditioned media from LNCaP and C4-2B cells preincubated with anti-VEGF neutralizing antibody decreased tube formation of HUVECs. These results suggest that VEGF secreted by prostate cancer cells is a potential inducer of HUVEC tube formation. Interestingly, conditioned media from LNCaP and C4-2B cells treated with 10 μmol/L of B-DIM induced less tube formation of HUVECs, and only the culture media containing 10 μmol/L of B-DIM had no such effects on tube formation within 6 h. Therefore, we speculate that decreased VEGF levels in conditioned media by B-DIM treatment are responsible for reduced tube formation. As predicted, we found that B-DIM decreased VEGF levels in conditioned media from LNCaP and C4-2B cells. Because VEGF is tightly regulated through transcriptional and posttranscriptional levels, we explore the transcriptional mechanism by which B-DIM may regulate VEGF. We did real-time RT-PCR using isoform-specific primers and probes and VEGF reporter gene assay. We showed that B-DIM inhibited VEGF transcription in LNCaP cells but not in C4-2B cells. Houck et al. indicated that VEGF bioavailability could be dually regulated at the genetic level by

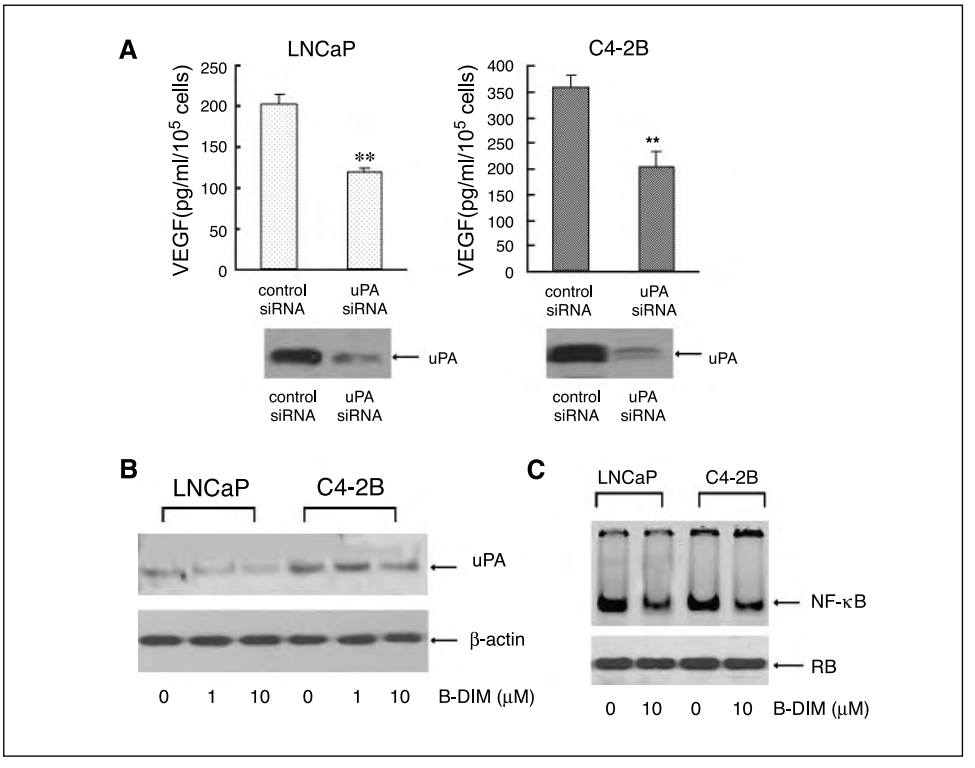

Figure 5. uPA regulated the release of VEGF and B-DIM repressed uPA expression and NF-κB DNA binding activity. *A,* LNCaP and C4-2B cells were transfected with uPA siRNA or control siRNA. The media were removed after 18 h transfection, and the cells were incubated in serum-free media for 24 h. The culture media were collected, centrifuged to remove cellular debris, and stored at −70°C until assay for VEGF. The cells were counted and cell lysates were subjected to gel electrophoresis, and Western blotting was done using anti-uPA antibody. *B,* LNCaP and C4-2B cells were seeded at a density of 2×10^5 per well in six-well plate. After 24 h, the cells were cultured in serum-free medium and incubated for another 24 h, and then treated with B-DIM or DMSO as vehicle control in 1% FBS for 24 h. Cell lysates from LNCaP and C4-2B cells were subjected to gel electrophoresis, and Western blotting was done using anti-uPA antibody. β-Actin protein was used as loading control. *C,* EMSA was done by incubating 3 μg of nuclear protein extracts from LNCaP and C4-2B cells treated with 10 μmol/L B-DIM or DMSO as vehicle control in 1% FBS for 24 h with IRDye 700–labeled NF-κB oligonucleotide. Retinoblastoma protein was used as a loading control. **, $P < 0.01$ compared with control siRNA. $n = 4$.

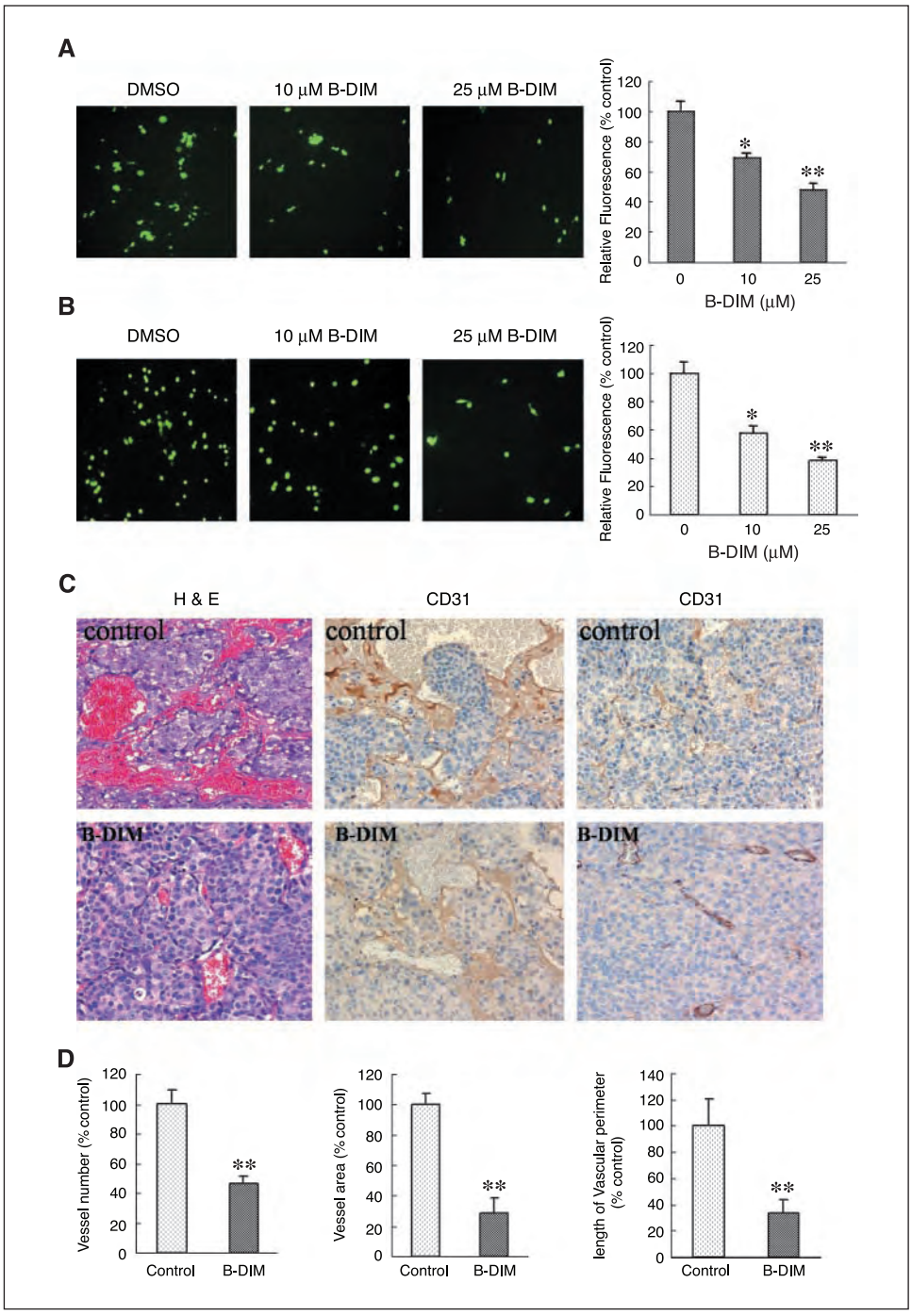

Figure 6. B-DIM inhibited LNCaP and C4-2B cell invasion and *in vivo* angiogenesis. The effects of B-DIM treatment on cell invasion were determined by using BD BioCoat Tumor Invasion Assay System. LNCaP (*A*) and C4-2B (*B*) cells with serum-free media containing 10 and 25 μmol/L of B-DIM or DMSO were seeded into the upper chamber of the system. Bottom wells were filled with complete media. After 24 h incubation, the cells invaded through the Matrigel membrane were stained with 4 μg/mL Calcein AM. The fluorescently labeled cells were photographed under a fluorescence microscope. The fluorescence of the invaded cells was read at excitation/emission wavelengths of 530/590 nm. *C,* C4-2B cells (4×10^6 in 50 μL PBS admixed with 0.25 mL Matrigel) were injected s.c. into the bilateral flanks of each mouse. The control group of mice was gavaged with the vehicle (sesame oil), whereas the other group of mice was gavaged with B-DIM (5 mg/kg body weight, daily) for 4 wks. On termination, Matrigel plugs with tumor cells were harvested and fixed in 10% formalin, dehydrated, and embedded in paraffin. Matrigel plug slices were stained with H&E (*left*) and anti-CD31 antibody (*middle* and *right*). SPOT Advanced Imaging software was used to capture bright-field images. Histologic pictures of peripheral (*middle*) and deeper (*right*) areas of Matrigel plugs. Original magnification (×200). *D,* image analyses of the area of CD31-positive vascular, vascular number, and length of vascular perimeter in each of the entire field were carried out using software image analysis program. *, $P < 0.05$; **, $P < 0.01$ compared with control $n = 4$.

alternative splicing of VEGF mRNA that determines whether VEGF will be soluble or incorporated into a biological reservoir and later activated through proteolysis after plasminogen activation (14). It is well known that uPA and MMP-9 can release bound-VEGF and regulate VEGF bioavailability. In this study, we found that B-DIM repressed the secretion of MMP-9 and protein synthesis in both cell lines and reduced the active uPA levels in LNCaP and C4-2B cells. These results suggest that B-DIM might inhibit tube formation of HUVECs induced by VEGF secreted from prostate cancer cells. In LNCaP cells, B-DIM decreased the bioactive VEGF by repressing VEGF gene transcription and reducing the bioavailability of VEGF bound to HSPGs in the ECM and cell membrane, which is released due to degradation of ECM by MMP-9 and cleavage by uPA. However, in C4-2B cells, B-DIM suppressed the bioactive VEGF mainly by reducing the bioavailability of VEGF via decreasing uPA and MMP-9 activity.

Increasing evidence suggests that MMP-9 contributes to the formation of microenvironment conducive to promote tumor growth and angiogenesis through increasing the association of VEGF and VEGFR2 (23, 28). MMP-9 can release bound VEGF from ECM via its proteolytic activity. Bergers et al. reported that the regulation of VEGF in the extracellular environment is implicated in the angiogenic switch. They found that MMP-9 could render normal islets angiogenic releasing VEGF, whereas MMP-9 inhibitor

could reduce angiogenic switching and tumor growth (29). A recent study has shown that VEGF bioavailability is regulated by MMPs through intramolecular processing, in which a subset of MMPs including MMP-9 could cleave matrix-bound isoforms of VEGF that release soluble fragments. Furthermore, MMP-cleaved VEGF has a distinct role in vascular patterning in tumors (15). In the current study, we found that B-DIM inhibited the secretion of MMP-9 and protein synthesis in both cell lines. MMP-9 transfections significantly increase VEGF levels from conditioned media compared with transfection of control plasmid in LNCaP and C4-2B cells. Importantly, 10 μmol/L of B-DIM treatment for LNCaP and C4-2B cells transfected with MMP-9 plasmid dramatically reduced the release of VEGF. Western blot analysis showed that B-DIM repressed MMP-9 expression in LNCaP and C4-2B cells transfected with MMP-9 plasmid, which indicated that B-DIM may regulate the MMP-9 expression through posttranscriptional mechanism. In addition, MMP-9 plasmid was constructed by inserting MMP-9 ORF into pcDNA3.1 vector. MMP-9 expression is driven by the CMV immediate/early promoter and enhancer containing many transcription factor binding sites, such as AP2, NF-κB, and cAMP-responsive element binding protein (30). In this study, we found that B-DIM inhibited NF-κB DNA binding activity. Thus, B-DIM may repress the MMP-9 expression in cells transfected with MMP-9 plasmid via inhibiting NF-κB DNA binding activity. Taken together, these results suggest that B-DIM decreases the secretion of VEGF and reduces the tube formation induced by tumor cells via inhibiting MMP-9 expression, leading to decreased bioavailability of the VEGF.

uPA is a highly specific serine protease which catalyzes plasmin formation from plasminogen. uPA, directly or via plasmin formation, leads to the release or activation of many angiogenic growth factors, such as bFGF and VEGF. Plouet et al. showed that uPA was able to cleave the VEGF189 without interaction with heparansulfate or sulfate modifications of the core proteoglycan into a 40 kDa isoform with more active mitogen (13). Therefore, VEGF189 released by MMP-9 via degradation of the ECM could be a substrate for uPA, and as such uPA, together with MMP-9, plays important roles in regulating the bioavailability of the VEGF. Plouet et al. also found that maturation of VEGF189 by uPA cleavage acquires the ability to activate KDR/FlK-1 (VEGFR-2). Native VEGF189 binds to Flt-1 (VEGFR-1) but not to VEGFR-2 (13). VEGFR-1 does not seem to transduce mitogenic signals in endothelial cells (31). uPA cleaves native VEGF189 into a diffusible isoform of 40 kDa with strong mitogenic activity which is able to activate VEGFR-2 and induce proliferation (13). In this study, we found that down-regulation of uPA by siRNA could reduce the release of VEGF. We also found that B-DIM inhibited uPA and MMP-9 expression, which protected native VEGF189 bound to HSPGs from cleavage by uPA, thereby inhibiting the mitogenic activity of VEGF for the proliferation of HUVECs.

NF-κB transcription factor is constitutively activated in prostate cancer cells and has been shown to contribute to the development and/or progression of prostate cancer (25). Moreover, angiogenesis, invasion, and metastasis are necessary for the development and progression of prostate cancer. Increasing evidence suggests that inhibition of NF-κB activity could suppress the angiogenesis, invasion, and metastasis by down-regulating the expression of NF-κB downstream target genes, such as VEGF, uPA, and MMP-9 (32, 33). NF-κB is normally retained in the cytoplasm in an inactive form, bound by inhibitory proteins called inhibitors of κB (IκB) in the most resting cells. NF-κB activation involves its release from its inhibitor and its subsequent translocation from the cytoplasma to the nucleus, where it binds to cognate sequences in the promoter region of many target genes. Therefore, NF-κB DNA binding activity is a hallmark for its activation. In this study, we found that B-DIM treatment inhibited the NF-κB DNA binding activity and decreased the expression of NF-κB downstream target genes, such as VEGF, MMP-9, and uPA, leading to the reduction of HUVEC tube formation *in vitro* and *in vivo* angiogenesis induced by the decreased VEGF secreted from prostate cancer cells and thereby inhibited LNCaP and C4-2B cell invasion. In a previous study, we found that DIM reduced phosphorylation of IκBα and blocked the translocation of p65, a subunit of NF-κB to the nucleus. DNA binding analysis and transfection studies with IκB kinase cDNA revealed that overexpression of IκB kinase mediates IκBα phosphorylation, which activates NF-κB,. and this activation was completely abrogated by DIM treatment in breast cancer cells (34). However, the exact mechanism by which B-DIM regulates the NF-κB activity, resulting in the inhibition of angiogenesis in prostate cancer, remains to be further investigated.

In summary, our results suggest that B-DIM reduces the bioavailability of VEGF by inhibiting NF-κB DNA binding activity leading to decreased MMP-9 and uPA expression, resulting in the inhibition of angiogenesis and invasion of prostate cancer cells. Compared with the inhibition of tube formation of HUVECs, B-DIM exhibits more inhibitory activity on angiogenesis *in vivo*, suggesting that B-DIM not only reduces the bioavailability of VEGF but also inhibits paracrine signaling of VEGF on endothelial cells *in vivo.* These results provide strong evidence in support of the antiangiogenic activity of B-DIM *in vivo*, which could contribute to the antitumor activity of B-DIM as previously supported by our laboratory in an animal model of prostate cancer (26).

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Review

Pancreatic cancer: Pathogenesis, prevention and treatment

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Abstract

Pancreatic cancer is the fourth leading cause of cancer death in the United States with a very low survival rate of 5 years. To better design new preventive and/or therapeutic strategies for the fight against pancreatic cancer, the knowledge of the pathogenesis of pancreatic cancer at the molecular level is very important. It has been known that the development and the progression of pancreatic cancer are caused by the activation of oncogenes, the inactivation of tumor suppressor genes, and the deregulation of many signaling pathways among which the EGFR, Akt, and NF-κB pathways appear to be most relevant. Therefore, the strategies targeting EGFR, Akt, NF-κB, and their downstream signaling could be promising for the prevention and/or treatment of pancreatic cancer. In this brief review, we will summarize the current knowledge regarding the pathogenesis, prevention, and treatment of pancreatic cancer.

Keywords: Pancreatic cancer; Molecular pathogenesis; EGFR; NF-κB; COX-2; Akt

Contents

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Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States with a median survival of <6 months and a dismal 5-year survival rate of 4.6% (Jemal et al., 2006). Even for those patients diagnosed with local disease, the 5-year survival rate is only 16%. Approximately, 33,730 people are expected to develop pancreatic cancer, and 32,300 people will die from the disease in 2006 (Jemal et al., 2006). The lethal nature of pancreatic cancer stems from its propensity to rapidly disseminate to the lymphatic system and distant organs. The presence of occult or clinical metastases at the time of diagnosis together with the lack of effective chemotherapies contributes to the high mortality in patients with pancreatic cancer. Pancreatic cancer is one of the most intrinsically drug-resistant tumors and the cancer cell resistance to chemotherapeutic agents is a major cause of treatment failure in pancreatic cancer. Therefore, there is a dire need for designing new and targeted therapeutic strategies that can overcome the drug-resistance and improve the clinical outcome for patients diagnosed with pancreatic cancer. For this purpose, the knowledge on the molecular pathogenesis of pancreatic cancer is very important and is likely to be helpful in the design of newer drugs and the molecular selection of existing drugs for targeted therapy against pancreatic cancer. The following sections will summarize what we know regarding the molecular pathogenesis of pancreatic cancer and how some of these molecular pathways could be exploited for the prevention and/or treatment of pancreatic cancer.

Molecular pathogenesis of pancreatic cancer

Intensive investigation of molecular pathogenesis will aid in identifying useful molecules for diagnosis, treatment, and prognosis of pancreatic cancer. In the past several years, considerable research has focused on identifying molecular events in pancreatic carcinogenesis, and their correlation with clinicopathological status. It has been found that multiple subsets of genes undergo genetic changes, either activation or inactivation, during the development and progression of pancreatic cancer (Mimeault et al., 2005). The activation of oncogenes and the inactivation of tumor suppressor genes are partly responsible for the initiation and progression of pancreatic cancers (Fig. 1) (Mimeault et al., 2005; Jimeno and Hidalgo, 2006; Maitra et al., 2006). Moreover, the deregulation of molecules in several cell signaling pathways, such as EGFR, Akt, NF-κB, etc., and their molecular cross talk (Fig. 1) also play important roles in the molecular pathogenesis of pancreatic cancer (Jimeno and Hidalgo, 2006; Mimeault et al., 2005).

Activation of oncogenes

Oncogenes can be activated through different mechanisms including point mutation and amplification. The activation of the *ras* oncogene has been found in more than 90% of pancreatic cancers (Almoguera et al., 1988). The *ras* gene family encodes a 21-kDa membrane-bound protein involved in signal transduction and mediates pleiotropic effects including cell proliferation and migration. Activated *ras* is involved in

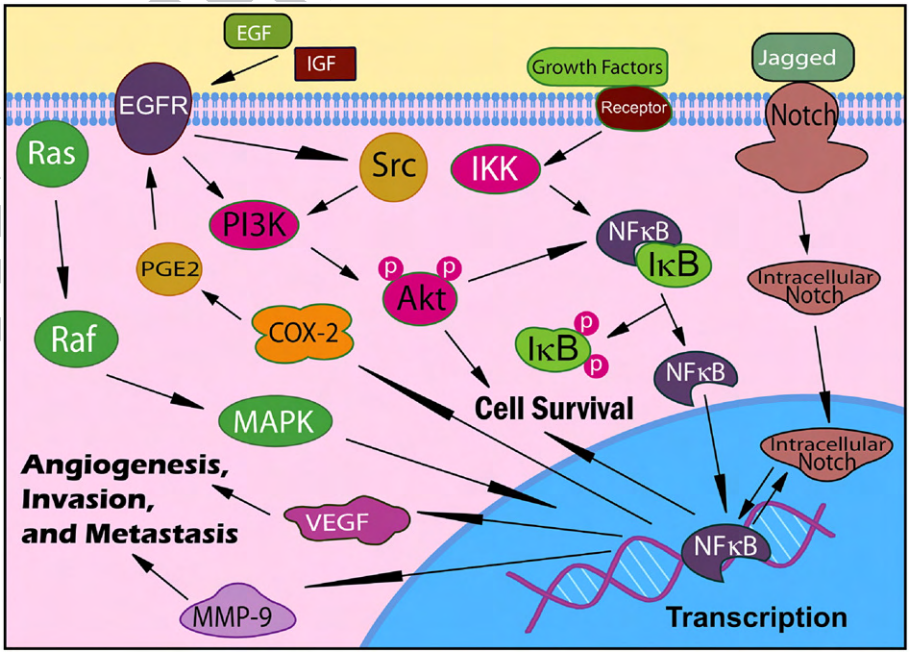

Fig. 1. The major cell signaling pathways involved in the pathogenesis of pancreatic cancer.

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growth factor-mediated signal transduction pathways. It has been found that approximately 80–90% of pancreatic cancers harbor point mutation at codons 12, 13 and 61 in *K-ras* (Almoguera et al., 1988). This is the highest fraction of *K-ras* alteration found in any human tumor type. The point mutation leads to the generation of a constitutively active form of *ras*. The constitutively activated *ras* binds to GTP and gives uncontrolled stimulation signals to downstream signaling cascades, promoting uncontrolled cell growth. *K-ras* mutation in pancreatic cancer typically develops during the early phase of carcinogenesis and patients with *K-ras* mutation have a shorter survival time than patients with the wild-type *K-ras*, suggesting that the mutation of *K-ras* is partly responsible for the initiation and progression of pancreatic cancer. In addition to point mutation, amplification of *ras* is also frequently observed in pancreatic cancers, suggesting that activation of *ras* oncogene is an important molecular event in pancreatic cancers. Moreover, recent studies have suggested the role of other genes such as *Notch* and *COX-2* in pancreatic cancers as discussed below.

Notch gene has also been considered as an oncogene involved in the pathogenesis of pancreatic cancer (Miyamoto et al., 2003). So far, four *Notch* genes have been identified (*Notch-1*, *Notch-2*, *Notch-3*, and *Notch-4*) and five Notch ligands (Dll-1, Dll-3, Dll-4, Jagged-1, and Jagged-2) have been found in mammals. Notch protein can be activated by interacting with its ligands. Upon activation, Notch protein is cleaved, releasing intracellular *Notch* which translocates into the nucleus. The intracellular *Notch* associates with transcriptional factors, which regulate the expression of target genes, and thus plays an important role in both organ development and pancreatic carcinogenesis. Therefore, it is clear that alterations in *Notch* signaling are associated with carcinogenesis of the pancreas. We and others have found that *Notch* signaling is frequently deregulated in human pancreatic cancers (Wang et al., 2006e, 2006a; Buchler et al., 2005). High expression of *Notch*, which inhibits apoptosis, has been found in pancreatic cancers. Moreover, *Notch-1* has been found to strongly induce the activity of NF-κB and the activation of NF-κB has been detected in the majority of pancreatic cancer, suggesting that the activation of *Notch* oncogene plays important roles in the pathogenesis of pancreatic cancer by activation of NF-κB and its downstream signaling pathways (Fig. 1).

The cyclooxygenase (COX) enzymes promote the formation of prostaglandins, which leads to the induction of cell growth. There are two isoforms of the COX enzyme. COX-1 is produced at a constant rate and the prostaglandins formed are involved in several normal physiologic events. COX-2, in contrast, is an inducible enzyme, and is not usually present in most normal tissues. However, its synthesis is stimulated in inflammatory and carcinogenic processes by cytokines, growth factors, and other cancer promoters. COX-2 has been shown to be increased in a variety of cancers including pancreatic cancers. Several pancreatic cancer cell lines strongly express COX-2. Immunohistochemical studies have shown that 47–66% of human pancreatic cancers over-express COX-2 and that COX-2 mRNA expression is much higher in tumors than in normal surrounding tissue (Okami et al., 1999). Moreover, there is a positive association between *ras* mutation and COX-2 level because activated *ras* acts to increase the stability of COX-2 mRNA. Therefore, COX-2 appears to be of significance in pancreatic carcinogenesis and as such there appears to be a cross talk between *ras*, *NF-κB*, *Notch* and *Cox-2* in cellular signaling that contributes to the molecular pathogenesis of pancreatic cancer (Fig. 1).

Amplification of other oncogenes also plays a very important role in the development and progression of pancreatic cancer. It has been found that *Akt-2* gene is amplified in 10–15% of pancreatic cancers while *Myb* gene is amplified in 10% of pancreatic cancers. The amplification of the oncogenes results in its activation and contributes to the stimulation of cell growth and the progression of pancreatic cancers. The experimental evidences also showed up-regulation of other oncogenes including *Src*, *Bcl-6*, *S100P*, *cyclin D1*, etc. (Mimeault et al., 2005). Up-regulation of *cyclin D1* has been found in pancreatic cancers and over-expression of *cyclin D1* is associated with poor prognosis. Inhibiting *cyclin D1* in pancreatic cancer cell lines leads to growth inhibition and loss of tumorigenicity in nude mice (Kornmann et al., 1998). Recent studies by our laboratory have shown that overexpression of *cyclin D1* promotes tumor cell growth and confers resistance to cisplatin-mediated apoptosis in an elastase-*myc* transgene-expressing pancreatic tumor cell line, suggesting the effect of *cyclin D1* on the progression of pancreatic cancer (Biliran et al., 2005).

In addition to these genes discussed above, no other oncogenes are known to play any significant or major role in pancreatic carcinogenesis; however, a group of tumor suppressor genes are also known to contribute in the molecular pathogenesis of pancreatic cancer as discussed below.

Inactivation of tumor suppressor genes

Inactivation of tumor suppressor genes is another important event for the initiation of pancreatic cancer. Tumor suppressor genes can be activated by mutation, deletion, or hypermethylation. The tumor suppressor genes targeted in pancreatic cancer include *p16*, *p53*, *SMAD4*, *PTEN*, etc.

It has been known that *p16* inhibits the activity of cyclin D and CDK4/6 complex. CDK4 and CDK6 normally interact with cyclin D to phosphorylate the retinoblastoma (Rb) protein. The phosphorylation of Rb allows it to dissociate from a complex formed with elongation factor 2 (E2F), allowing E2F to activate genes required for DNA synthesis in cell cycle. *p16* controls cell cycle progression through G1/S transition by inhibiting cyclin D and CDK4/6 mediated phosphorylation of Rb, inhibiting cell growth. Approximately 95% of pancreatic cancer patients have inactivated *p16* (40% deletion; 40% mutation; 15% hypermethylation) in the tumors (Schutte et al., 1997). Experimental studies have demonstrated that transfection of wild-type *p16* into human pancreatic cancer cells results in decreased tumor cell proliferation *in vitro* and *in vivo*. Moreover, in pancreatic cancer patients, the tumor size is significantly larger and the survival time is significantly shorter with *p16* mutation compared to patients with wild-type *p16*. These evidences demonstrate that *p16* alterations participate in the aggressiveness of pancreatic cancer through its interaction with various cellular signaling pathways.

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In addition to *p16*, another tumor suppressor gene, *p53*, is well known to be involved in the control of cell cycle. *p53* binds to the $p21^{WAF1}$ promoter and stimulates the production of $p21^{WAF1}$, which negatively regulates the complex consisting of cyclin D1 and CDK2, thereby arresting the cell at the G1 phase and inhibiting cell growth. The *p53* also plays important roles in the induction of apoptotic cell death. Inactivation of *p53* during carcinogenesis can lead to uncontrolled cell growth and increased cell survival. The *p53* gene is inactivated in about 50% of pancreatic cancers through gene mutation and deletion. We have shown that *p53* mutation results in alteration of the 3D structure of the p53 protein (Li et al., 1998). The *p53* mutation and the alteration of the protein 3D structure have been associated with shorter survival in patients with pancreatic cancer. In addition, alterations in the *p53* gene are associated with *K-ras* mutations, suggesting a cross talk and cooperative activity between *p53* and *K-ras* in the molecular pathogenesis of pancreatic cancers. Moreover, the status of the *p53* is important in mediating the cancer cell specific effects of chemotherapeutic agents. Loss of *p53* function could result in decreased sensitivity to certain types of chemotherapeutic agents. Therefore, the status of *p53* could be a useful guide for those patients who are likely to respond to adjuvant chemotherapy.

The inactivation of *DPC4* (deleted in pancreatic cancer locus 4, Smad4) tumor suppressor gene is another common genetic alteration identified in pancreatic cancer. The *DPC4* gene encodes for a 64-kDa protein, Smad 4, which plays roles in the inhibition of cell growth and angiogenesis. The inactivation of *DPC4* is relatively specific to pancreatic cancer although it occurs with low incidence in other cancers. It has been found that *DPC4* tumor suppressor gene is deleted in approximately 50% of pancreatic cancers (Cowgill and Muscarella, 2003). Up to 90% of pancreatic adenocarcinomas could harbor loss of heterozygosity. *DPC4* alterations occur relatively late in pancreatic carcinogenesis. The frequency of loss of *DPC4* expression is significantly higher in poorly differentiated pancreatic adenocarcinoma and the pancreatic cancer patients with intact *DPC4* gene have significantly longer survival after resection compared to the patients with mutant *DPC4* gene. Furthermore, *DPC4* inactivation is always accompanied by the inactivation of *p16*, suggesting its importance in the pathogenesis of pancreatic cancer.

As indicated earlier, $p21^{WAF1}$ is an inhibitor of CDK. It forms complexes with cyclinA/CDK2 or cyclinD1/CDK4 and inhibits their activity, causing cell cycle arrest in G1 phase. Loss of $p21^{WAF1}$ activity has been observed in approximately 30–60% of pancreatic cancers (Garcea et al., 2005). $p27^{CIP1}$ is another CDK inhibitor which regulates cell cycle progression from G1 to S phase. The loss of $p27^{CIP1}$ expression has also been observed in pancreatic cancers (Garcea et al., 2005). Another tumor suppressor gene, *BRCA2*, has been found to participate in DNA damage repair and mutations in *BRCA2* have been linked to a significantly increased risk of pancreatic cancer. These evidences demonstrate that inactivation of $p21^{WAF1}$, $p27^{CIP1}$, and *BRCA2* tumor suppressor genes is involved in the pathogenesis of pancreatic cancer. Although these genes as discussed above play important roles in pancreatic cancer, the role of these genes in cellular signaling is still poorly understood. Below we will discuss several important signaling pathways that are known to play significant roles and as such became important target for pancreatic cancer therapy.

Deregulation of EGFR signaling

EGFR consists of an extracellular ligand-binding domain, a hydrophobic transmembrane region, and an intracellular tyrosine kinase domain. EGFR is a member of the ErbB family of receptor tyrosine kinases, which include ErbB-1 (EGFR), ErbB-2 (HER-2), ErbB-3, and ErbB-4. The principal ligands of EGFR are EGF and TGF-α. Binding of a ligand to EGFR induces receptor dimerization, which results in intracellular transphosphorylation of tyrosine residues. Phosphorylation of EGFR activates molecules in different cell signaling pathways including PI3K, Src, MAPK, STAT, etc., inducing cell cycle progression, cell division, survival, motility, invasion, and metastasis.

The genomic alterations of EGFR that occur in cancers include over-expression, mutation, deletion, and rearrangement. These alterations of EGFR induce the activity of receptor tyrosine kinases and may promote the development and progression of pancreatic cancer. Experimental studies have shown that EGFR activation plays important roles in proliferation, apoptosis inhibition, angiogenesis, metastasis, and resistance to chemotherapy or radiation therapy. Overexpression of EGF and EGFR is a common feature of human pancreatic cancer (Talar-Wojnarowska and Malecka-Panas, 2006). HER-2/neu amplification and p185 overexpression have been observed in 60% of pancreatic cancers. Moreover, EGFR overexpression has been found significantly more often in advanced clinical stages of pancreatic cancer and thus is associated with shorter survival in pancreatic cancer patients (Talar-Wojnarowska and Malecka-Panas, 2006), suggesting that deregulation of the EGFR pathway participates in the development and progression of pancreatic cancer. The EGFR signaling and its downstream signaling is therefore an important signaling pathway targeted for pancreatic cancer therapy.

Deregulation of Akt signaling

It has been known that EGF binding and subsequent EGFR, ras, or Src activation lead to the activation of the PI3K pathway. Activated PI3K phosphorylates phosphatidylinositides (PIP3), which then phosphorylates and activates Akt. Phosphorylated Akt (p-Akt) promotes cell survival by inhibiting apoptosis and activating NF-κB. It has been known that p-Akt inhibits apoptosis through its ability to phosphorylate and inactivate several targets including Bad, Forkhead transcription factors, and caspase-9, all of which are involved in the apoptotic pathway. Akt also regulates the NF-κB pathway via phosphorylation and activation of molecules in the NF-κB pathway, suggesting that there is a cross talk between these two signaling pathways.

It has been found that a significant proportion (46–70%) of pancreatic cancer has high levels of p-Akt, which is correlated with high tumor grade and poor prognosis. Inhibition of PI3K in

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pancreatic cell lines results in a decreased p-Akt, G1 cell cycle arrest, and reduced cell proliferation, suggesting that PI3K/Akt pathway plays important roles in the survival of pancreatic cancer cells. Moreover, the inhibition of Akt decreases the function of NF-κB, and has been shown to sensitize Mia-PaCa-2 pancreatic cancer cells to chemotherapy (Fahy et al., 2004), suggesting that both Akt and NF-κB are targets for the treatment of pancreatic cancers.

Deregulation of NF-κB signaling

The NF-κB signaling pathway plays important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling (Karin, 2006). In human cells without specific signal, NF-κB is sequestered in the cytoplasm through tight association with its inhibitors: IκBα which acts as a NF-κB inhibitor and p100 proteins which serves as both an inhibitor and precursor of NF-κB DNA-binding subunits. NF-κB can be activated through phosphorylation of IκBα by IKKβ and/or phosphorylation of p100 by IKKα, leading to degradation of IκBα and/or the processing of p100 into small form (p52). This process allows two forms of NF-κB (p50–p65 and p52–RelB) to become free, resulting in the translocation of active NF-κB into the nucleus for binding to NF-κB-specific DNA-binding sites and, in turn, regulating gene transcription. By binding to the promoters of target genes, NF-κB controls the expression of many genes (i.e. survivin, MMP-9, uPA, VEGF, etc.) that are involved in cell survival, apoptosis, invasion, metastasis, and angiogenesis.

NF-κB is constitutively activated in most human pancreatic cancer tissues and cell lines but not in normal pancreatic tissues and cells, suggesting that the activation of NF-κB is involved in the carcinogenesis of pancreatic cancers. Inhibition of NF-κB by a super-inhibitor of NF-κB results in impaired proliferation and induction of apoptosis (Liptay et al., 2003), suggesting an important role of NF-κB in pancreatic tumorigenesis. Moreover, it has been found that the inhibition of constitutive NF-κB activity completely suppresses the liver metastasis of the pancreatic cancer cell line ASPC-1 (Fujioka et al., 2003). An experimental study also demonstrates that urokinase-type plasminogen activator (uPA), one of the critical proteases involved in tumor invasion and metastasis, is over-expressed in pancreatic cancer cells, and its over-expression is induced by constitutive NF-κB activity (Wang et al., 1999). These results suggest that constitutively activated NF-κB is tightly related to the invasion and metastasis frequently observed in pancreatic cancers. Moreover, the deregulation of NF-κB could also be due to Notch signaling as discussed earlier and as such the cross talk between Notch and NF-κB appears to be an important signaling event that regulates the processes of tumor invasion and angiogenesis in pancreatic cancer.

Deregulation of Hedgehog and other signaling

Hedgehog (Hh) signaling is an essential pathway for embryonic pancreatic development. It has been known that Hh signaling plays important roles in proper tissue morphogenesis and organ formation during the developing gastrointestinal tract. Hedgehog ligands including sonic hedgehog (Shh) are expressed throughout the endodermal epithelium at early embryonic stages but excluded from the region that forms the pancreas. Deregulation of the Hh pathway has been implicated in a variety of cancers including pancreatic cancer. It has been reported that overexpression of Shh may contribute to pancreatic tumorigenesis. Thayer et al. found that no Shh was detected in the islets, acini, or ductal epithelium of normal pancreas while shh was aberrantly expressed in 70% of specimens from the patients with pancreatic adenocarcinoma, suggesting that Shh is a mediator of pancreatic cancer tumorigenesis (Thayer et al., 2003). Importantly, the down-regulation of Shh by cyclopamine, a specific inhibitor of Shh, can reduce the growth and viability of pancreatic cancer cells, suggesting that targeting Shh signaling may be an effective novel approach for the treatment of pancreatic cancer (Berman et al., 2003). A recent report shows that NF-κB contributes to Hh signaling pathway activation through Shh induction in pancreatic cancer (Nakashima et al., 2006), demonstrating the cross talk between NF-κB and Hh signaling pathways in pancreatic cancer.

The deregulation of other signaling pathways (i.e. STAT3, MAPK, VEGF, IGF, etc.) also plays roles in the development and progression of pancreatic cancer. Therefore, oncogenes, tumor suppressor genes, and the complex interactions between many cellular signaling pathways make up a network whose cross talk contributes to the molecular pathogenesis of pancreatic cancer, suggesting that targeted inactivation of these important signaling pathways could be a novel and newer approach for the prevention and/or treatment of pancreatic cancer.

Prevention of pancreatic cancer progression by dietary chemopreventive agents

It has been estimated that more than two-thirds of human cancers could be prevented by modification of lifestyle including dietary modification. The dietary factors which are associated with increased risk of pancreatic cancer are meat, red meat in particular, and energy. Protection is mainly provided by fruit, vegetables, and vitamins. In recent years, more dietary compounds have been recognized as cancer chemopreventive agents because of their anti-carcinogenic activity. Therefore, early invasion and metastasis of pancreatic cancer could be preventable by these dietary compounds.

Soy isoflavone, genistein

Genistein is the main isoflavone found in a relatively high concentration in soybeans and most soy-protein products. It has been found that genistein inhibits cell growth and induces apoptosis in various cancers including pancreatic cancer. Our laboratory has investigated whether genistein treatment could modulate NF-κB DNA binding activity in pancreatic cancer cells (Li et al., 2004b, 2005a; Banerjee et al., 2005). We have

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found that genistein treatment significantly inhibited NF-κB DNA-binding activity in pancreatic cancers *in vitro* and *in vivo*. We have also investigated the effect of genistein on the Akt signaling pathway. We have found that genistein also inhibited Akt activity in pancreatic cancer cells (Banerjee et al., 2005; Li et al., 2005a). Furthermore, we found that genistein significantly down-regulated Notch signaling, leading to the inhibition of NF-κB and induction of apoptosis in pancreatic cancer cells (Wang et al., 2006e, 2006d). Our data together with others have revealed that genistein inhibits carcinogenesis and cancer progression through inhibition of Akt, Notch, and NF-κB activation, resulting in the inhibition of their downstream genes as depicted in Fig. 1. These results suggest that genistein could be a potent agent for the prevention and/or treatment of pancreatic cancer progression. In addition to genistein, there are other attracting agents that could also be useful for pancreatic cancer as discussed below.

I3C and DIM

Indole-3-carbinol (I3C) is produced from naturally occurring glucosinolates contained in a wide variety of plants including members of the family Cruciferae. Vegetables of the genus *Brassica* in the family Cruciferae contribute to most of our intake of glucosinolates and include all kinds of cabbages, broccoli, cauliflower, and brussels sprouts. I3C is biologically active and it is easily converted *in vivo* to its dimeric product 3,3′-diindolylmethane (DIM), which is also biologically active. It has been found that both I3C and DIM inhibit cell proliferation and induces apoptotic cell death in a variety of cancers including pancreatic cancer (Abdelrahim et al., 2006). It has been reported that 13C induces apoptosis in pancreatic cancer cells through the inhibition of STAT3 whose activation has been observed in human pancreatic carcinoma specimens and pancreatic cell line but not in normal pancreatic tissues (Lian et al., 2004), suggesting that I3C and DIM could have some beneficial effects on pancreatic cancer.

Curcumin

Curcumin is a compound from *Curcuma longa* (tumeric). *C. longa* is a plant widely cultivated in tropical regions of Asia and Central America. Curcumin has recently received considerable attention due to its pronounced anti-inflammatory, anti-oxidative, immunomodulating, anti-atherogenic, and anti-carcinogenic activities. It has been found that curcumin suppress the activation of NF-κB through inhibition of IKK activity in pancreatic cancer cells (Li et al., 2004a). By inhibition of NF-κB, curcumin also down-regulates the expression of COX-2, resulting in increased PGE_2 and activation of several cytokines and chemokines especially IL-8. We have also found that curcumin inhibits cell growth and induces apoptosis through down-regulation of Notch and NF-κB signaling in pancreatic cancer (Wang et al., 2006c), suggesting that curcumin could be useful for the prevention and/or treatment of pancreatic cancer similar to soy isoflavone genistein.

EGCG

Consumption of green tea has been implicated in better human health including the prevention of cancers. Green tea contains several catechins including epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epigallocatechin-3-gallate (EGCG). However, EGCG has been believed to be the most potent for the inhibition of oncogenesis and reduction of oxidative stress among these catechins (Mukhtar and Ahmad, 1999). EGCG has been shown to inhibit the formation and the growth of solid tumors in laboratory animals. By targeting multiple signaling pathways including MAPK, EGFR and NF-κB, EGCG is able to inhibit the malignant transformation of epidermal cell lines, to inhibit cell growth, and to induce apoptosis in a number of cancer cells including pancreatic cancer (Mukhtar and Ahmad, 1999; Khan et al., 2006; Qanungo et al., 2005). EGCG also selectively inhibits COX-2 without affecting COX-1 expression (Hussain et al., 2005) and down-regulates *K-ras* (Lyn-Cook et al., 1999), suggesting its effects on the inactivation of oncogenes. Furthermore, the treatment of PANC1, Mia-PaCa-2, and BxPC-3 pancreatic cell lines with EGCG caused significant suppression of the invasive ability of the pancreatic cancer cells (Takada et al., 2002). These reports provide strong evidence in support of the roles of EGCG in chemoprevention and/or treatment of pancreatic cancer, especially because EGCG targets important cell signaling molecules as depicted in Fig. 1.

Resveratrol

Resveratrol is a phytoalexin present in a wide variety of plant species including grapes, mulberries, and peanuts. Relatively high quantities of resveratrol are found in grape juice and red wine. Resveratrol has been shown to have beneficial effects on the reduction of oxidative stress and the prevention of cancers. Resveratrol was first noted to be a cancer chemopreventive agent having antioxidant and anti-tumorigenic properties (Jang et al., 1997). Like EGCG, it is a polyphenol which can cause G1 cell cycle arrest in various tumor cell lines including pancreatic cancer (Ding and Adrian, 2002). Resveratrol can activate protein kinases such as Jun N-terminal kinase, resulting in phosphorylation and activation of p53. Resveratrol can also induce apoptosis independent of p53 status. Moreover, resveratrol-induced apoptosis was found to be associated with the inhibition of NF-κB activity in pancreatic cell lines, suggesting its potential chemopreventive activity against pancreatic cancer.

Lycopene and vitamins

Tomato products including ketchup, tomato juice, and pizza sauce, are the richest sources of lycopene in the US diet. It has been reported that dietary intake of lycopene is associated with reduced pancreatic cancer risk, suggesting its role in the prevention of pancreatic cancer (Nkondjock et al., 2005). However, the role of lycopene in pancreatic cancer has been very limited.

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The sources of vitamin C are fruits and vegetables, particularly orange, strawberry, citrus, kiwi, and cauliflower. Studies using rats in which pancreatic lesions have been induced with azaserine have shown that a diet high in vitamin C results in reduced tumor formation (Woutersen et al., 1999). It has been reported that a reduced risk for pancreatic cancer is associated with higher intake of vitamin C and D (Skinner et al., 2006; Lin et al., 2005). These limited studies provide little evidence in support of their roles in pancreatic cancer prevention.

The above results especially soy isoflavones, I3C, curcumin, EGCG, and resveratrol appear to target similar signaling pathways all of which are known to be involved in the development and progression of pancreatic cancers and are important targets for pancreatic cancer prevention and/or treatment. It is important to note that primary prevention of pancreatic cancer is not feasible due to lack of identifiable risk factors for the development of pancreatic cancer. However, existing knowledge provides sufficient information as to the novel application of several dietary or nutritional agents for the prevention of pancreatic cancer progression. In addition, these agents could be also useful for the treatment of pancreatic cancer either as single agents or in combination, especially in combination with existing therapeutic agents as discussed in the following paragraphs.

New strategies for the treatment of pancreatic cancers

Targeting EGFR pathway for enhancing cancer therapeutic efficacy

Two classes of EGFR inhibitors, monoclonal antibodies and small molecule tyrosine kinase inhibitors have been considered for the treatment of pancreatic cancer. Cetuximab, a monoclonal antibody targeting EGFR, binds to EGFR competitively with high affinity, preventing activation of EGFR by its ligands. By binding to EGFR, cetuximab inhibits cell proliferation, enhances apoptosis, and reduces angiogenesis and invasion (Marshall, 2006). Cetuximab is currently under investigation in pancreatic cancer. The combination of cetuximab and gemcitabine showed promising activity against advanced pancreatic cancer, and improved survival in animal study (Bruns et al., 2000) and clinical trial (Xiong et al., 2004). Trastuzumab, an anti-HER-2/neu antibody, also showed cell growth inhibitory activity against human pancreatic cancer cell lines and anti-tumor activity in orthotopic mouse model.

Erlotinib (Tarceva) is a small molecule inhibitor of the EGFR tyrosine kinase. Experimental studies have shown that erlotinib inhibits EGFR tyrosine kinase activity and cell growth in pancreatic cancer cell lines and in an animal model (Marshall, 2006). In a phase III trial for patients with advanced pancreatic cancer, erlotinib plus gemcitabine has shown a statistically significant survival benefit compared with gemcitabine alone (Tang et al., 2006). In another study combining erlotinib with gemcitabine and irradiation for locally advanced unresectable pancreatic cancer, most of the evaluable patients showed disease stabilization (Iannitti et al., 2005). When combined with capecitabine, erlotinib has shown considerable anti-tumor activity and better tumor control in a phase II trial for advanced pancreatic cancer. These results suggest that targeting EGFR signaling could be useful for the treatment of pancreatic cancer. Because of the importance of EGFR in pancreatic cancer, we were also interested in finding newer ways to block EGFR signaling and our efforts resulted in the identification and characterization of a novel negative regulator of EGFR, termed EGFR-related protein (ERRP), whose expression was found to attenuate EGFR activation.

We have also found that ERRP significantly inhibits cell proliferation and induces apoptosis in BxPC-3, HPAC, and PANC-1 pancreatic cancer cells (Zhang et al., 2005, 2006). ERRP also inhibits ligand-induced activation of EGFR, HER-2, and HER-3. Most importantly, ERRP was found to inhibit pancreatic tumor growth in a SCID mouse xenograft model. The anti-tumor activity of ERRP correlated well with down-regulation of NF-κB, MAPK, Akt, and Notch-1 (Wang et al., 2006b; Zhang et al., 2005, 2006). ERRP also down-regulates NF-κB downstream genes such as *VEGF* and *MMP-9*, and inhibits cancer cell invasion, suggesting that ERRP could be a very potent agent for the treatment of pancreatic cancer by inhibiting cell survival signaling, tumor growth, invasion, and angiogenesis. These results suggest that further development of ERRP for human application is warranted.

Targeting COX-2 for enhancing cancer therapeutic efficacy

As indicated earlier, COX-2 and its metabolic product (PGE_2) play important roles in pancreatic cancer, suggesting that targeting COX-2 could have a therapeutic benefit against pancreatic cancer. Several COX-2 inhibitors have shown their activity in reducing tumor growth with different mechanisms. Indomethacin, one of the COX-2 inhibitors, inhibits both isoforms of the COX enzyme while newer agents, such as celecoxib, inhibit only COX-2 and are more desirable for clinical use. In an orthotopic pancreatic cancer animal model, celecoxib treatment showed inhibition of tumor growth, angiogenesis, and metastasis (Wei et al., 2004). Nonsteroidal anti-inflammatory drugs (NSAIDs) also show their inhibitory effect on COX-2 and PGE_2, leading to a reduced incidence of tumor formation and a reduced number of tumors per animal. Experiments using two NSAIDs, sulindac and NS398, in pancreatic cancer cell lines, have shown that both agents cause a dose-dependent inhibition of cancer cell growth.

A combination of the agents, celecoxib and Zyflo (a 5-lipoxygenase inhibitor), has shown a reduction in the incidence and size of pancreatic tumors, and a reduced number of liver metastases in animal model. We have previously reported that celecoxib potentiates gemcitabine-induced growth inhibition through the down-regulation of NF-κB activation and induction of apoptosis in pancreatic cancer cells (El-Rayes et al., 2004). In a clinical trial, the patients with advanced pancreatic adenocarcinoma were given celecoxib in combination with 5-fluorouracil. The results showed that celecoxib with 5-fluorouracil was capable of inducing durable and objective

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responses, even in gemcitabine-resistant pancreatic cancer (Milella et al., 2004). These results collectively suggest that COX-2 inhibition with other novel agents could be useful in future clinical trials for pancreatic cancer. The concept of extracellular signaling leading to the activation of internal signaling in the pathogenesis of pancreatic cancer has been well recognized. These signaling cascades that lead to the activation of membrane signaling, such as Akt signaling, could be a novel therapeutic target for pancreatic cancer treatment as discussed below.

Targeting Akt to enhance cancer therapeutic efficacy

Akt pathway is another important cell signaling pathway involved in drug resistance. It has been found that genistein enhanced necrotic-like cell death due to the significant inhibition of Akt activity in cancer cells treated with genistein and adriamycin, suggesting that the enhanced growth inhibition of combination treatment is through the inactivation of the Akt pathway (Satoh et al., 2003). Reports from our laboratory and others have also showed that activated Akt is inhibited by genistein combined with gemcitabine or radiation in pancreatic and other cancer cells, suggesting that enhancement of chemotherapeutic or radiation effects by genistein may be partially mediated by the Akt pathway (Yashar et al., 2005; Banerjee et al., 2005). Bava et al. reported that curcumin down-regulated Taxol-induced phosphorylation of Akt, which interacts with NF-κB, suggesting that enhanced anti-tumor activity by curcumin is through the inactivation of Akt and NF-κB pathways (Bava et al., 2005). These results clearly suggest that many chemopreventive agents as discussed earlier are potent inhibitors of Akt and NF-κB signaling pathways. In addition, it has been reported that a small molecule inhibitor, QLT0254, inhibits tumor growth through the PI3K/Akt pathway and enhances gemcitabine-induced apoptosis in human orthotopic primary pancreatic cancer xenografts (Yau et al., 2005). These results suggest that down-regulation of Akt can enhance cancer therapeutic efficacy. The studies from our laboratory and others have shown that Akt is directly linked with the activation of NF-κB and as such may represent a novel approach for targeting both Akt and NF-κB for the prevention and/or treatment of pancreatic cancer.

Targeting NF-κB for enhancing cancer therapeutic efficacy

De novo resistance to chemotherapeutic agents is a major cause of treatment failure in pancreatic cancer, which could be due to the constitutive activation of NF-κB among others. Moreover, chemotherapeutic agents can activate NF-κB in pancreatic and other cancer cells, leading to cancer cell resistance to chemotherapy (acquired resistance). Therefore, the strategies by which NF-κB could be inactivated represent a novel approach for the treatment of pancreatic cancer.

The *in vitro* and *in vivo* studies from our laboratory and others have demonstrated that the anti-tumor effects of chemotherapeutic agents can be enhanced by combination treatment with genistein through inhibition of NF-κB. By *in vitro* and *in vivo* studies, we have found that NF-κB activity was significantly increased by cisplatin, docetaxel, doxorubicin, and gemcitabine treatment and that the NF-κB-inducing activity of these agents was completely abrogated by genistein pre-treatment in pancreatic cancer cells, suggesting that genistein pre-treatment inactivates NF-κB and may contribute to increased growth inhibition and apoptosis induced by these agents (Banerjee et al., 2005; Li et al., 2004b, 2005a; Mohammad et al., 2006). Recently, we found that anti-tumor and anti-metastatic activities of docetaxel could be enhanced by genistein through regulation of osteoprotegerin/receptor activator of NF-κB (RANK)/RANK ligand/MMP-9 signaling *in vitro* and *in vivo* (Li et al., 2006). Other investigators have also reported similar results showing that the inhibition of NF-κB contributes to the sensitization of pancreatic cancer cells to chemotherapeutic agents (Muerkoster et al., 2003; Zhang et al., 2003).

We have found that I3C and DIM inhibit cancer cell growth and induce apoptosis through inhibition of NF-κB (Rahman and Sarkar, 2005; Li et al., 2005b). We and others have also found that combinations of I3C and cisplatin or tamoxifen inhibit the growth of cancer cells more effectively than either agent alone (Sarkar and Li, 2004; Cover et al., 1999), suggesting that I3C and DIM could potentiate the activity of chemotherapeutic agents by down-regulation of NF-κB, which appears to be a central and important target for the treatment of pancreatic cancer.

Using gene therapy to enhance cancer chemotherapeutic efficacy

In recent years, gene therapy based on the knowledge of molecular pathogenesis has been actively developed as a novel therapeutic strategy to be used alone or in combination with conventional chemotherapy in pancreatic cancer. Strategies for gene therapy in pancreatic cancer include antisense and RNA interference strategies whereby the function of activated oncogenes (*K-ras*, *H-ras*, *Notch*, *LSM1*, etc.) is inhibited, and strategies to restore the function of tumor suppressor genes (*p53*, *p16*, *p21*, *SMAD4*, etc.). These therapeutic strategies have shown their promising effects on the inhibition of pancreatic cancer *in vitro* and *in vivo* (Bhattacharyya and Lemoine, 2006). In addition, gene-directed pro-drug activation therapy is another gene therapy system in which a gene that encodes an enzyme is delivered to tumor cell. Then, a pro-drug administered as chemotherapy is metabolized by the enzyme to release cytotoxic drug at the site of the tumor. A recent report has shown that introduction of a fusion gene combining deoxycytidine kinase and uridine monophosphate kinase, which convert gemcitabine into its toxic phosphorylated metabolite, sensitizes pancreatic cancer cells to gemcitabine by reducing dramatically both *in vitro* cell viability and *in vivo* tumor volume (Vernejoul et al., 2006). Moreover, the combinations of gemcitabine treatment with introduction of IFN-α, NK4, or p53 significantly suppress the growth and the metastasis of human pancreatic cancer cells, suggesting the enhancement of chemotherapy by gene therapy approach in pancreatic cancer (Bhattacharyya and Lemoine,

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2006; Ogura et al., 2006). Collectively, the brief information presented in this article provides state-of-the-art knowledge toward new and novel therapies for pancreatic cancer and as such must be tested in human patients.

Conclusion

The development and progression of pancreatic cancer are closely associated with the activation of oncogenes, the inactivation of tumor suppressor genes, the deregulation of EGFR, Akt, NF-κB, and their downstream signaling pathways. Therefore, novel and newer strategies targeting EGFR, NF-κB, COX-2, and Akt signaling pathways to interrupt their molecular cross talk (Fig. 1) could be promising for the prevention and/or treatment of pancreatic cancer.

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Regulation of FOXO3a/β-catenin/GSK-3β signaling by 3,3'-diindolylmethane (DIM) contributes to inhibition of cell proliferation and induction of apoptosis in prostate cancer cells*

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Running Title: Regulation of FOXO3a/GSK-3β/β-catenin by B-DIM

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Previous studies from our laboratory have shown anti-proliferative and pro-apoptotic effects of 3,3'-diindolylmethane (DIM) through regulation of Akt and androgen receptor (AR) in prostate cancer cells. However, the mechanism by which DIM regulates Akt and AR singling pathways has not been fully investigated. It has been known that FOXO3a and GSK-3β, two targets of activated Akt, interact with β-catenin, regulating cell proliferation and apoptotic cell death. More importantly, FOXO3a, GSK-3β, and β-catenin all are AR coregulators and regulate the activity of AR, mediating the development and progression of prostate cancers. Here, we investigated the molecular effects of B-DIM, a formulated DIM with higher bioavailability, on Akt/FOXO3a/GSK-3β/β-catenin/AR signaling in hormone-sensitive LNCaP and hormone-insensitive C4-2B prostate cancer cells. We found that B-DIM significantly inhibited the phosphorylation of Akt and FOXO3a and increased the phosphorylation of β-catenin, leading to the inhibition of cell growth and induction of apoptosis. We also found that B-DIM significantly inhibited karyopherin, one of the nuclear importins, binding to β-Catenin, suggesting an inhibitory effect of B-DIM on β-Catenin nuclear translocation. By EMSA and ChIP assay, we found that B-DIM inhibited FOXO3a binding to the promoter of AR and promoted FOXO3a binding to $p27^{KIP1}$ promoter, resulting in the alteration of AR and $p27^{KIP1}$ expression, the inhibition of cell proliferation, and the induction of apoptosis in both androgen sensitive and insensitive prostate cancer cells. These results suggest that B-DIM induced cell growth inhibition and apoptosis induction are partly mediated through the regulation of Akt/FOXO3a/GSK-3β/β-Catenin/AR signaling. Therefore, B-DIM could be a promising non-toxic agent for possible treatment of hormone-sensitive but most importantly hormone-refractory prostate cancers.

Prostate cancer has become a significant health problem in the United States because of its high incidence and mortality (1). Despite an initial efficacy of androgen-deprivation therapy, most patients with prostate cancer progress from androgen-dependent status to hormone-refractory prostate cancer (HRPC) for which there is no curative therapy. It is still unclear how the prostate cancer cells progress to androgen-independence. However, more evidences indicate that androgen receptor (AR) and Akt pathways participate in the development of HRPC (2;3).

It has been known that during the progression of prostate cancers from androgen sensitive status to an androgen independent stage, the majority of prostate cancer cells still expresses AR, suggesting that AR signaling plays a critical role in the development and progression of prostate cancer (4). In prostate epithelial cells, ligand-free AR is sequestered in the cytoplasm bound to heat shock proteins (HSPs). Binding of androgen to the AR induces AR conformational change, which allows dissociation of HSPs. The AR then forms a homo-dimer and is phosphorylated. This phosphorylation stabilizes

the ligand-AR complex and the complex translocates to the nucleus (5). The activated AR then initiates gene transcription by binding to specific androgen-response elements in the promoter regions of target genes. Evidence is emerging showing that phosphorylation of the AR by other molecules in cell signaling pathways can also influence AR transactivation (6). It has been found that Akt can phosphorylate the AR at Ser210/213 and Ser790/791 and transactivate the activity of AR (7;8). The phosphorylation by Akt also sensitizes the AR to low circulating levels of androgen, such as those present during maximum androgen blockade (9). This sensitization allows low levels of androgen to induce phosphorylation at specific sites required for translocation of the AR to the nucleus. Therefore, Akt is another activator of AR required for androgen-independent survival and growth of prostate cancer cells.

Akt can be activated by the lipid kinase phosphatidylinositol-3 kinase (PI3K). The activated Akt can inhibit apoptosis by direct phosphorylation of its downstream molecules. Two of the important targets of Akt-mediated phosphorylation are the Forkhead transcription factor class O (FOXO) and glycogen synthase kinase 3 (GSK-3). The family of FOXO mainly includes FOXO1, FOXO3a, and FOXO4. By binding to the nuclear importer, active FOXO3a translocates to the nucleus, binds to DNA, and promotes the transcription of its target genes, inducing either cell cycle arrest or apoptosis (10). However, activated Akt regulates transcription of FOXO3a target genes through modulation of FOXO3a activity by phosphorylating it at three conserved serine/threonine residues (Thr32, Ser253, and Ser315), leading to the release of FOXO3a from DNA and translocation to the cytoplasm. Interestingly, recent report shows that FOXO3a also promotes the transcription of AR (11). Therefore, Akt/FOXO3a/AR signaling appears to play important roles in the development of hormone-refractory prostate cancer.

GSK-3, including GSK-3α and GSK-3β, has been implicated in a variety of physiological processes such as induction of apoptosis. GSK-3β also controls cell survival through regulation of β-catenin, one of the important molecules in Wnt signaling. In the nucleus, β-catenin acts as a transcriptional coactivator and activates genes involved in cell proliferation and survival. GSK-3β binds to β-catenin and induces phosphorylation of β-catenin, which is subsequently ubiquitinated and degraded via the protosome pathway within the cytoplasmic compartment (12). The activity of GSK-3β can be inhibited through phosphorylation at Ser9 by activated Akt, resulting in more nonphosphorylated β-catenin moving into the nucleus. Importantly, GSK-3β and β-catenin are two important AR co-regulators, which regulate transcriptional activity of AR and play important roles in the development of clinical androgen escape by regulation of AR oncogenic functions (3;13;14). Moreover, both GSK-3β and β-catenin can interact with molecules (including IKK, p105, etc) in the NF-κB cell signaling pathway (12;15;16), regulating cell survival. Therefore, Akt/GSK-3β/β-Catenin/AR signaling may play important roles in the development of HRPC and specific targeting of these signaling pathways could be important for killing HRPC cells.

Studies from our laboratory and others have found that 3,3'-diindilylmethane (DIM) shows potent anti-proliferative activities against various cancers including prostate cancer (17-19). We have also reported that B-DIM, a formulated DIM with higher bioavailability *in vivo* (20), induces growth inhibition and apoptotic cell death in both androgen-sensitive LNCaP and androgen-insensitive C4-2B prostate cancer cells through the down-regulation of AR and Akt (21). However, no studies have been reported to date to elucidate the effect and molecular mechanisms of action of B-DIM on FOXO3a/GSK-3β/β-Catenin signaling in prostate cancer cells, especially in HRPC cells. Here we report, for the first time, that B-DIM is a potent agent for inducing growth inhibition and apoptotic cell death in both androgen-sensitive LNCaP and androgen-insensitive C4-2B prostate cancer cells partly mediated through the inactivation of Akt/FOXO3a/GSK-3β/β-Catenin/AR signaling pathways.

EXPERIMENTAL PROCEDURES

Cell lines, reagents, and antibodies - Human prostate cancer cell lines including LNCaP and C4-2B cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37^{o}C. B-DIM

(BioResponse, Boulder, CO; known as BR-DIM and referred as B-DIM) was generously provided by Dr. Michael Zeligs and was dissolved in DMSO to make a 50 mM stock solution. IGF-1 was purchased from R&D Systems (Minneapolis, MN) and LY294002 was from Sigma (St. Louis, MO). Anti-AR (Santa Cruz, Santa Cruz, CA), anti-Akt (Santa Cruz), anti-pAkt Ser473 (Cell Signaling, Danvers, MA), anti-FOXO3a (Cell Signaling), anti-pFOXO3a Ser253 (Cell Signaling), anti-pFOXO3a Thr32 (Cell Signaling), anti-β-Catenin (Cell Signaling), anti-p-β-Catenin Ser33/37/Thr41 (Cell Signaling), anti-GSK-3 (Upstate), anti-Karyopherin α (BD Biosciences, San Diego, CA), anti-Karyopherin β (BD Biosciences), and anti-β-actin (Sigma) primary antibodies were used for immunoprecipitation, Western Blot analysis, or immunofluorescent staining.

Cell proliferation inhibition studies by MTT assay - Human LNCaP and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 hours, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium supplemented with 100 ng/ml IGF-1, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 hours. Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were incubated with MTT (0.5 mg/ml, Sigma) in medium at 37°C for 2 hours and then with isopropanol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 595 nm.

Histone/DNA ELISA for detection of apoptosis - The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in prostate cancer cells treated with B-DIM according to the manufacturer's protocol. Briefly, LNCaP and C4-2B cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 hours as described above. The cytoplasmic histone/DNA fragments from these cells were then extracted and incubated in microtiter plate modules coated with anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments followed by color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 405 nm.

Preperation of cytoplasmic and nuclear lysates - LNCaP and C4-2B cells were treated with 50 μM B-DIM for 48 and 72 hours. After treatment and harvesting, the cells were resuspended in lysis buffer (0.08 M KCl/ 35 mM HEPES pH 7.4/ 5 mM K-phosphate pH 7.4/ 5 mM $MgCl_2$/ 25 mM $CaCl_2$/ 0.15M sucrose/ 2mM PMSF/ 8mM DTT) and frozen at –80°C overnight. The cell suspension was thawed and passed through a 28 gauge needle three times. A small aliquot of the cells were checked for cell membrane breakage using Trypan Blue. Then, the cell suspension was centrifuged and the supernatant were saved as cytoplasmic lysate. The pellet (nuclei) was suspended in lysis buffer and the nuclei were lysed by sonication. After centrifugation, supernatant was saved as nuclear lysate. The protein concentration in each sample was measured using Coomassie Plus Protein Assay (Pierce, Rockford, IL).

Immunoprecipitation - Nuclear lysate (500 μg) and cytoplasmic lysate (800 μg) were diluted and subjected to immunoprecipitation using 2.5-5 μg of anti-AR, anti-FOXO3a, or anti-β-catenin antibody. The lysate and antibody mixtures were incubated overnight at 4°C with rotation. After adding 50 μl of Protein G Agarose (Santa Cruz) and incubation for 1 hour, the samples were centrifuged. The agarose pellet was then washed three times, resuspended in 50 μl of Laemmli buffer with 2-mercaptoethanol, and boiled for 5 minutes. Boiled samples were centrifuged and supernatant was used for Western Blot analysis.

Western Blot analysis - LNCaP and C4-2B cells were treated with 50 μM B-DIM for 24-72 hours. Whole cell lysate of the cells was prepared by sonicating the cell lysed in 62 mM Tris-HCl and 2% SDS. The protein concentration was measured by BCA Protein Assay (Pierce). Immunoprecipitates and whole cell lysates were subjected to 10% or 14% SDS-PAGE and

electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with indicated primary antibodies, and subsequently incubated with appropriate secondary antibody conjugated with peroxidase (Bio-rad, Hercules, CA) or TrueBlot secondary antibody (eBioscience, San Diego, CA). The signal was then detected using the chemiluminescent detection system (PIERCE) and quantified with AlphaEaseFC software (Alpha Innotech, San Leandro, CA)

Immunofluorescence staining - LNCaP and C4-2B cells were plated on cover slips in each well of a 6-well plate. Cells were then treated with 50 μM B-DIM for 24-72 hours. Cells were then fixed with acetone for 15 minutes, rinsed with PBS, and incubated with 1% BSA in PBS for 30 minutes. The cells were then incubated with anti-FOXO3a or anti-β-catenin antibody at 4°C overnight. After washing with PBS, the cells were incubated with FITC-conjugated (1:100, Santa Cruz) or Texas Red-conjugated (1:100, Santa Cruz) secondary antibody at 37°C for 1 hour and washed with PBS. Cell images were observed under a fluorescent microscope.

Electrophoretic mobility shift assay (EMSA) - EMSA was conducted to measure the activity of FOXO3a binding to AR or $p27^{KIP1}$ promoter in B-DIM treated and untreated cells. LNCaP and C4-2B cells were treated as described above. Following treatment, cells were harvested and incubated in ice-cold cell lysis buffer (10 mM HEPES pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine) on ice. After 15 minutes, NP-40 was added to the cell suspension at a final concentration of 0.3% and the samples were vortex vigorously for 20 seconds. After centrifugation, the supernatant was saved as cytoplasmic protein and the nuclear pellet was incubated in ice-cold nuclear extraction buffer (20 mM HEPES pH7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.5 mg/ml benzamidine) on ice for 30 minutes. After centrifugation, the supernatant was saved as nuclear protein and protein concentration was measured using BCA Protein Assay (PIERCE). 8 μg of nuclear protein was assembled with 10 mM Tris-HCl, 50 mM NaCl, 6 mM DTT, 0.5% Tween 20, 0.08 mg/ml poly(dI):poly(dC), and 0.01 μM IRDye 700 or 800-labeled oligonucleotide (LI-COR, Lincoln, NE) which contains AR or $p27^{KIP1}$ promoter sequences with consensus FOXO3a DNA binding site. The sequences of oligonucleotides are as follows: 5'-ATTATGTCCTTGTTTCAGCCTGTT-3' for AR; 5'-ATGTGTAGCTTGTTTTCTTAGCCAC-3' for $p27^{KIP1}$ (11;22;23). After incubation at room temperature for 30 minutes, the samples were loaded on a pre-run 4.5% (for AR) or 8% (for $p27^{KIP1}$) polyacrylamide gel and electrophoresis was continued at 100v for 60 min. The signal was then detected and quantified by using Odyssey Infrared Imaging System (LI-COR). Super shift assay using FOXO3a antibody was also conducted to confirm the specificity of FOXO3a DNA-binding activity.

Chromatin immunoprecipitation (ChIP) assay - ChIP assay was performed to test the effect of B-DIM on FOXO3a binding to AR or $p27^{KIP1}$ promoter under an *in vivo* environment. Briefly, LNCaP and C4-2B cells were treated with 50 μM B-DIM for 48 hours. Formaldehyde was then added to the cells at a final concentration of 1%. After formaldehyde cross-linking reaction was quenched with 125 mM glycine, the cells were incubated in cell lysis buffer (5 mM HEPES pH 8.0, 85 mM KCl, 0.5% NP-40, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF) on ice for 10 minutes. The nuclei were pelleted by centrifugation and resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). After incubation on ice for 10 minutes, the nuclear lysates were sonicated to generate an average DNA size of ~600 bp and protein concentration was measured using BCA Protein Assay (PIERCE). 1000 μg of each sample were diluted and 200 μg of diluted sample were kept as input. The remaining sample was incubated with FOXO3a antibody (Santa Cruz) at 4°C overnight. The FOXO3a antibody-bound AR and $p27^{KIP1}$ promoter DNA complexes were pelleted with Protein G Agarose at 4°C for 2 h. After washing and elution, the immunoprecipitated complexes and input were reversed from cross-linking by adding RNase and 0.3 M NaCl and heating at 65°C for 5 hours. The input DNA and

immunoprecipitated DNA were then precipitated with ethanol. After treated with proteinase K and purified with phenol/chloroform, the purified DNA was precipitated again with ethanol. Real-time and conventional PCRs were performed using purified DNA (input DNA for β-actin and FOXO3a immunoprecipitated DNA for AR or $p27^{KIP1}$ promoter with FOXO3a binding site) and the following primers: AR promoter (forward, 5'-AGCCTTTCCCAAATGACAATG-3'; reverse, 5'-GCACAGCCAAACATCATAGG-3'), $p27^{KIP1}$ promoter (forward, 5'-GTCCCTTCCAGCTGTCACAT-3'; reverse, 5'-GGAAACCAACCTTCCGTTCT-3'), β-actin (forward, 5'-CCACACTGTGCCCATCTACG-3'; reverse, 5'-AGGATCTTCATGAGGTAGTCAGTCAG-3'). For real-time PCR, data were analyzed according to the comparative Ct method and the amount of FOXO3a-bound AR promoter DNA was normalized by input β-actin DNA. For conventional PCR, PCR product was visualized by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Inhibition of FOXO3a phosphorylation by B-DIM - We previously reported that B-DIM significantly inhibited the phosphorylation of Akt and the expression of AR in LNCaP and C4-2B prostate cancer cells (21). To further investigate how B-DIM suppresses the expression of AR through the inhibition of Akt phosphorylation, we tested the effects of B-DIM on FOXO3a, one of the main downstream target genes of Akt. We observed that phosphorylation of Akt and FOXO3a was upregulated by IGF-1 and down-regulated by LY294002 in prostate cancer cells as expected (Fig. 1A and 1C). We found that B-DIM significantly inhibited the phosphorylation of FOXO3a in C4-2B and LNCaP cells (Fig. 1B). Moreover, B-DIM abrogated the up-regulation of Akt and FOXO3a phosphorylation induced by IGF-1 (Fig. 1A, 1B, and 1C). Furthermore, we found that B-DIM significantly inhibited the phosphorylation of FOXO3a at Thr32 and Ser253 and decreased the ratio of p-FOXO3a over FOXO3a in both the cytoplasm and nucleus of prostate cancer cells (Fig. 1C), suggesting that B-DIM could help to retain a greater amount of FOXO3a in activated form to inhibit cancer cell growth. By immunoprecipitation, we found that B-DIM significantly inhibited the formation of FOXO3a and Akt complex in the cytosol and nucleus of prostate cancer cells (Fig. 1D), consistent with Akt inactivation and subsequent decrease of FOXO3a phosphorylation by B-DIM treatment. We also found that B-DIM significantly decreased the formation of FOXO3a and AR complex which could inhibit FOXO3a activity in both cytoplasmic and nuclear components (Fig. 1D), suggesting that greater level of active FOXO3a proteins will exert their anti-proliferative and pro-apoptotic effects on B-DIM treated cells. It has been known that activated Akt also phosphorylates and inactivates GSK-3β, leading to the dephosphorylation of β-catenin and cancer cell survival. β-catenin also interacts with FOXO3a to regulate cell growth. Therefore, we further investigated the effects of B-DIM on GSK-3β/β-catenin signaling.

Increased GSK-3β binding to β-Catenin and induction of β-Catenin phosphorylation by B-DIM - By Western Blot analysis, we found that B-DIM significantly decreased the level of β-catenin protein in the cytosol and nucleus of C4-2B cells (Fig. 2A and 2B). More importantly, we found that B-DIM enhanced the phosphorylation of β-catenin and increased the ratio of p-β-catenin over β-catenin in the nucleus of C4-2B cells (Fig. 2B). By immunoprecipitation, we discovered that GSK-3β binding to β-catenin was increased upon B-DIM treatment (Fig. 2C), consistent with the increase in the phosphorylation of β-catenin. We also found that B-DIM inhibited FOXO3a binding to β-catenin (Fig. 2C). In LNCaP cells, we also observed similar effects of B-DIM on the down-regulation of β-catenin level and up-regulation of β-catenin phosphorylation (data not shown). These results suggest that B-DIM treatment leads to a greater level of inactivated β-catenin and less of activated β-catenin in prostate cancer cells, causing a decrease in the activated β-catenin in the nucleus of prostate cancer cells. Therefore, we further investigated the location of β-catenin and FOXO3a altered by B-DIM treatment.

Inhibition of β-Catenin nuclear localization by B-DIM - Indeed, we observed lower level of β-

catenin protein in the nucleus after B-DIM treatment (Fig. 2A and 2B). By immunofluorescent staining, we found that B-DIM significantly inhibited the nuclear localization of β-catenin; however, no significant change in the nuclear localization of FOXO3a was observed upon B-DIM treatment (Fig. 3A). To confirm these results, we also tested the expression of karyopherin, one of the nuclear importins, and the interaction of β-catenin with karyopherin in B-DIM treated and un-treated prostate cancer cells. We found that B-DIM significantly decreased the level of karyopherin protein in cytoplasm and nucleus of C4-2B cells (Fig. 3B). More importantly, we found that B-DIM significantly inhibited karyopherin binding to β-catenin, consistent with the data from immunofluorescent staining, demonstrating the inhibitory effects of B-DIM on the nuclear translocation of β-catenin. However, we did not observe such a significant inhibitory effect of B-DIM on the binding of karyopherin to FOXO3a (Fig. 3C). We also observed similar effects of B-DIM on the relocation of β-catenin in LNCaP cells (data not shown). Since FOXO3a as a transcription factor could regulate the transcription of its downstream genes including AR and $p27^{KIP1}$, we tested the effects of B-DIM on the transcriptional activity of AR and $p27^{KIP1}$ regulated by FOXO3a.

Inhibition of FOXO3a mediated AR transcription by B-DIM - By EMSA, we found that B-DIM inhibited the activity of FOXO3a protein binding to AR promoter DNA in C4-2B and LNCaP cells (Fig. 4A and 4B), suggesting the inhibitory effect of B-DIM on the FOXO3a induced transcription of AR. We also conducted ChIP assay with conventional and real-time PCR to confirm this effect of B-DIM in a live cell environment. We found that B-DIM treatment decreased FOXO3a protein binding to AR promoter *in vivo* in C4-2B (Fig. 4C and 4D) and LNCaP (data not shown) prostate cancer cells. By Western Blot analysis, we indeed observed the down-regulation of AR expression by B-DIM treatment (Fig. 1A), consistent with the results from EMSA and ChIP assay.

Stimulation of FOXO3a mediated $p27^{KIP1}$ expression by B-DIM - In contrast with AR, using EMSA we found that B-DIM significantly increased the activity of FOXO3a protein binding to $p27^{KIP1}$ promoter DNA in C4-2B cells (Fig. 5A). ChIP assay confirmed EMSA data, showing that B-DIM significantly enhanced FOXO3a protein binding to $p27^{KIP1}$ promoter *in vivo* in prostate cancer cells (Fig. 5B and 5C). Furthermore, we found increased level of $p27^{KIP1}$ protein after B-DIM treatment (Fig. 5D), consistent with the data from EMSA and ChIP assay. We also observed similar results in LNCaP cells (data not shown). Since FOXO3a, GSK-3β, and β-catenin all are important factors that regulate cell survival and apoptotic cell death, we tested the effects of B-DIM on cell proliferation and apoptosis.

Inhibition of cell proliferation and induction of apoptosis by B-DIM through regulation of Akt/FOXO3a/β-Catenin/GSK-3β/AR signaling - By MTT assay, we found that IGF-1, which activates Akt, promoted cell proliferation in LNCaP and C4-2B cells (Fig. 6A and 6B). Importantly, B-DIM inhibited cell proliferation and abrogated IGF-1 induced cell proliferation (Fig. 6A and 6B). By apoptosis assay, we observed that IGF-1 protected cancer cells from apoptosis (Fig. 6C and 6D). However, B-DIM induced apoptosis and abrogated IGF-1 mediated protection of LNCaP and C4-2B cells from apoptosis. These results are consistent with our observation on the alterations of Akt and FOXO3a caused by IGF-1 or B-DIM treatment (Fig. 1A and 1C), suggesting that the inhibition of cell proliferation and induction of apoptosis by B-DIM is partly mediated by the deregulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling pathways.

DISCUSSION

FOXO3a is a main molecule in the Akt signaling pathway. It plays important roles in the control of cell survival and apoptotic cell death through transcriptional regulation of genes which are critically involved in the processes of cell cycle and apoptosis (24). The main regulatory event that controls FOXO3a induced transcription is phosphorylation or dephosphorylation of FOXO3a. The ratio of p-FOXO3a over FOXO3a is one of the factors that determine whether cancer cells will survive or undergo apoptotic cell death.

From our series of studies, we found that B-DIM significantly inhibited the phosphorylation and activation of Akt, resulting in the lower levels of p-FOXO3a (Fig. 7). More importantly, B-DIM inhibited the phosphorylation of FOXO3a and abrogated the inactivation of FOXO3a induced by IGF-1 in both cytoplasmic and nuclear compartments, causing less p-FOXO3a and more FOXO3a in the nucleus compared to control. However, we did not find any significant change in FOXO3a binding to one of the nuclear import protein, karyopherin. These results suggest that B-DIM regulates the activity of FOXO3a through the regulation of FOXO3a phosphorylation rather than the regulation of FOXO3a nuclear importing. It has been known that FOXO3a regulates the transcription of $p27^{KIP1}$ and AR by binding to their promoters (11;25). Interestingly, using EMSA and ChIP assay, we found that B-DIM induced FOXO3a binding to $p27^{KIP1}$ promoter and suppressed FOXO3a binding to AR promoter, suggesting its effects on up-regulation of $p27^{KIP1}$ and down-regulation of AR. Indeed, we found that $p27^{KIP1}$ expression was increased and AR expression was decreased upon B-DIM treatment. Up-regulation of $p27^{KIP1}$ promoter activity and protein expression by B-DIM-mediated FOXO3a regulation could lead to the growth inhibition and apoptotic cell death that we have observed in prostate cancer cells. It has been reported that FOXO3a activates AR expression (11) while AR inactivates FOXO3a (26), forming a regulatory loop to maintain a balance between AR and FOXO3a. The decreased levels of AR protein and AR binding to FOXO3a as observed by Western Blot analysis and immunoprecipitation clearly suggest that B-DIM could interrupt the above mentioned regulatory loop, resulting in the inhibition of AR transactivation (Fig. 7). In addition, the alteration of $p27^{KIP1}$ protein level by B-DIM treatment could also be due to the altered activity of proteasome as observed in other agent treatment (27) and, as such, require further investigation.

β-catenin is an important molecule in Wnt signaling pathway (28). However, it also interacts with GSK-3β to regulate cell proliferation through activation of Akt signaling pathway. It has been known that activated Akt phosphorylates and inactivates GSK-3β, causing degradation of GSK-3β (29). If Akt is inactivated, GSK-3β can phosphorylate and inactivate β-catenin, leading to a decrease in the nuclear translocation of β-catenin (3). In most cancer cell lines, activated Akt causes more β-catenin to translocate to the nucleus and bind to T-cell factor (TCF), stimulating the expression of genes which are related to cell proliferation and survival (3;28;30). In this study, we found that B-DIM treatment increased GSK-3β binding to β-catenin, suggesting that B-DIM could up-regulate the phosphorylation of β-catenin. Indeed, we observed higher levels of p-β-catenin and lower levels of β-catenin in the nucleus of B-DIM treated C4-2B and LNCaP cells compared to control. Moreover, B-DIM decreased the protein level of karyopherin, which has been known to be a main nuclear import protein to carry important nuclear regulatory factors into the nucleus (31;32), suggesting that B-DIM could inhibit the nuclear translocation of nuclear regulatory factors. Indeed, we previously reported that B-DIM inhibited AR nuclear translocation (21). In the present study, we observed that β-catenin nuclear translocation was decreased upon B-DIM treatment. In the nucleus, β-catenin interacts with TCF and further regulates the transcription of genes in the Wnt signaling pathway (28;33). Therefore, our results clearly suggest that B-DIM could down-regulate Wnt signaling and inhibit cell survival through the regulation of GSK-3β/β-catenin signaling (Fig. 7).

It has been known that Akt, FOXO3a, β-catenin, and GSK-3β interact with each other to regulate cell survival and apoptosis (34-36). Moreover, all of them act as AR regulators or co-regulators that influence AR transactivation through regulation of phosphorylation and transcription of AR (11;13;14). It is obvious that there is significant crosstalk between Akt, FOXO3a, GSK-3β, β-catenin, and AR. Therefore, the Akt/FOXO3a/GSK-3β/β-catenin/AR signaling pathway appears to play critical roles in the control of cell growth and apoptosis. In the present study, we found that B-DIM elicits its effects on each of these molecules at every step of this signaling pathway. The effect of B-DIM could begin from the inhibition of Akt activation and cause subsequent series of alterations of signal transduction in this signaling pathway, finally leading to the inhibition of AR transactivation in both androgen sensitive and hormone refractory prostate cancers (Fig. 7). More importantly, it has been found that GSK-3β functions as a critical

regulator in the development of androgen independent prostate cancer by regulation of the oncogenic function of AR (3). In addition, β-catenin mediated Wnt signaling also interacts with AR and plays important roles in the development and progression of prostate cancer (37). We found that B-DIM could regulate these molecules, cause down-regulation of AR transactivation, and result in the decreased level of PSA as we reported previously (21), suggesting that B-DIM could be very effective for the treatment of prostate cancer.

It has been well known that Akt controls cell proliferation and apoptosis. Growth factor IGF-1 has been found to activate Akt (38;39), suggesting that IGF-1 could induce cell growth and inhibit apoptosis. Indeed, we found that IGF-1 treatment caused an increase in the amount of p-Akt, induced cell proliferation, and decreased the number of apoptotic cells. However, B-DIM abrogated the IGF-1 induced Akt activation and cell proliferation and caused more apoptotic cell death. B-DIM also affected Akt downstream genes including FOXO3a and GSK-3β due to inactivation of Akt, resulting in the down-regulation of both β-catenin and AR. These results suggest that B-DIM inhibited cell proliferation and induced apoptotic cell death partly through the regulation of Akt/FOXO3a/GSK-3β/β-catenin/AR signaling. Based on our molecular mechanistic pre-clinical data, we believe that B-DIM could be a promising non-toxic agent for the possible treatment of androgen-dependent but most importantly hormone-refractory prostate cancers.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. **B-DIM inhibited phosphorylation of Akt and FOXO3a.** A: C4-2B cells were treated with 100 ng/ml IGF-1, 20 μM LY294002, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 hours. Total lysate from each sample was subjected to Western Blot analysis. B: LNCaP and C4-2B cells were treated with 50 μM B-DIM for 24 and 48 hours. Cytoplasmic lysate from each sample was subjected to Western Blot analysis. C: C4-2B cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM plus 100 ng/ml IGF-1, or 20 μM LY294002 for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to Western Blot analysis. The ratio of p-FOXO3a over FOXO3a was calculated by standardizing the ratios of each control to the unit value. D: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Cytoplasmic and nuclear lysates from each sample were subjected to immunoprecipitation and Western Blot analysis (C: control; D: B-DIM treatment).

Figure 2. **B-DIM inhibited the expression of β-catenin and induced phosphorylation of β-catenin.** A: C4-2B cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM, or 20 μM LY294002 for 48 hours.

Total, cytoplasmic, and nuclear lysates from each sample were subjected to Western Blot analysis. The ratio of β-catenin over β-actin was calculated by standardizing the ratios of each control to the unit value. B: C4-2B cells were treated 50 μM B-DIM for 48 hours. Total lysate from each sample was subjected to Western Blot analysis (C: control; D: B-DIM treatment). The ratios of β-catenin over β-actin and p-β-catenin over β-catenin were calculated by standardizing the ratios of each control to the unit value. C: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Total lysates from each sample were subjected to immunoprecipitation and Western Blot analysis (C: control; D: B-DIM treatment).

Figure 3. **B-DIM inhibited the nuclear translocation of β-catenin but did not alter FOXO3a nuclear translocation.** A: C4-2B cells were treated with 50 μM B-DIM for 48 hours. The samples were subjected to immunofluorescent staining using anti-β-catenin (a and b) and anti-FOXO3a (c and d) antibodies. (a and c: control; b and d: B-DIM treatment). B, C, and D: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to immunoprecipitation and/or Western Blot analysis (C: control; D: B-DIM treatment; Cyto: cytoplasmic; Nu: nuclear). The ratio of Karyopherin α over β-actin was calculated by standardizing the ratios of each control to the unit value.

Figure 4. **B-DIM inhibited FOXO3a binding to the promoter of AR.** A and B: C4-2B (A) and LNCaP (B) cells were treated with 30 or 50 μM B-DIM for 24, 48, and 72 hours. Nuclear proteins from each sample were subjected to EMSA using AR oligonucleotide. (Arrow indicating the specific DNA binding. The density of each signal was calculated by standardizing each control to the unit value). C and D: C4-2B cells were treated with 50 μM B-DIM. The samples were then subjected to ChIP assay using real-time PCR (C; bars: mean±SE; n=3) or conventional PCR (D; ARP: using AR promoter primers; C: control; D: B-DIM treatment).

Figure 5. **B-DIM up-regulated FOXO3a binding to the promoter of $p27^{KIP1}$.** A: C4-2B cells were treated with 30 or 50 μM B-DIM for 24 and 72 hours. Nuclear proteins from each sample were subjected to EMSA using $p27^{KIP1}$ oligonucleotide. (Arrow indicating the specific DNA binding. The density of each signal was calculated by standardizing each control to the unit value). B and C: C4-2B cells were treated with50 μM B-DIM. The samples were then subjected to ChIP assay using real-time PCR (B; bars: mean±SE; n=3) or conventional PCR (C; p27P: using $p27^{KIP1}$ promoter primers; C: control; D: B-DIM treatment). D: C4-2B cells were treated with 50 μM B-DIM for 48 hours. Total, cytoplasmic, and nuclear lysates from each sample were subjected to Western Blot analysis (C: control; D: B-DIM treatment; Cyto: cytoplasmic; NE: nuclear extract).

Figure 6. **B-DIM inhibited cell growth and induced apoptosis.** A, B, C, and D: C4-2B and LNCaP cells were treated with 100 ng/ml IGF-1, 50 μM B-DIM, or 50 μM B-DIM plus 100 ng/ml IGF-1 for 48 hours. Cell proliferation was tested by MTT assay (A and B). Apoptotic cell death was tested by Cell Death Detection ELISA (C and D). *: $p<0.05$, compared to control; n=3).

Figure 7. **Schematic representation of molecular effect of B-DIM on Akt/FOXO3a/GSK-3β/β-catenin/AR signaling.**

Figure 1

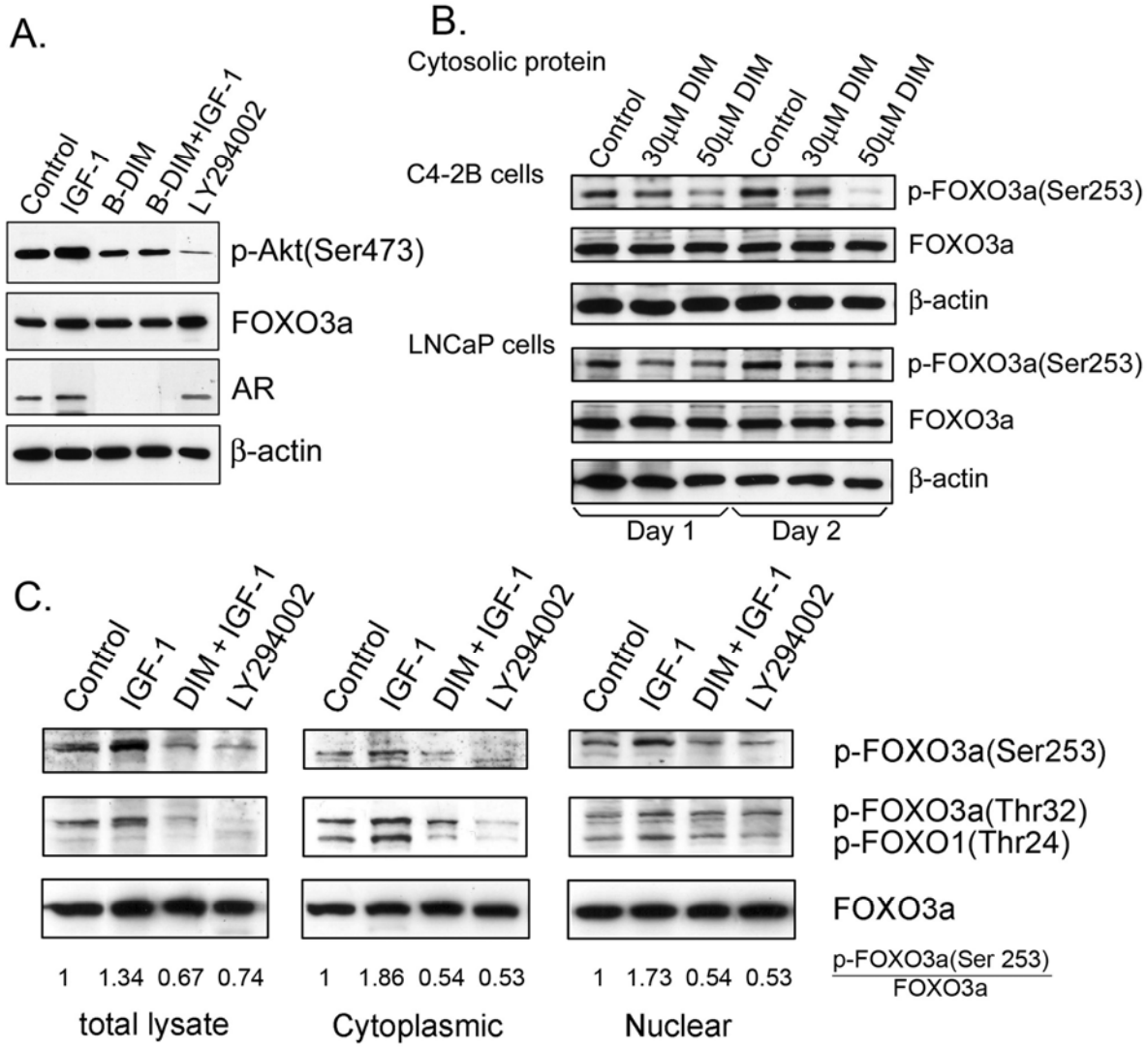

A.
Control
IGF-1
B-DIM
B-DIM+IGF-1
LY294002
p-Akt(Ser473)
FOXO3a
AR
β-actin
B.
Cytosolic protein
Control
30μM DIM
50μM DIM
Control
30μM DIM
50μM DIM
C4-2B cells
p-FOXO3a(Ser253)
FOXO3a
β-actin
LNCaP cells
p-FOXO3a(Ser253)
FOXO3a
β-actin
Day 1
Day 2
C.
Control
IGF-1
DIM+IGF-1
LY294002
p-FOXO3a(Ser253)
p-FOXO3a(Thr32)
p-FOXO1(Thr24)
FOXO3a
1 1.34 0.67 0.74
total lysate
1 1.86 0.54 0.53
Cytoplasmic
1 1.73 0.54 0.53
Nuclear
p-FOXO3a(Ser 253)
FOXO3a
D.
Nuclear
C D
IP: FOXO3a
Akt
AR
Cytosol
C D
IP: FOXO3a
Akt
AR

Figure 2

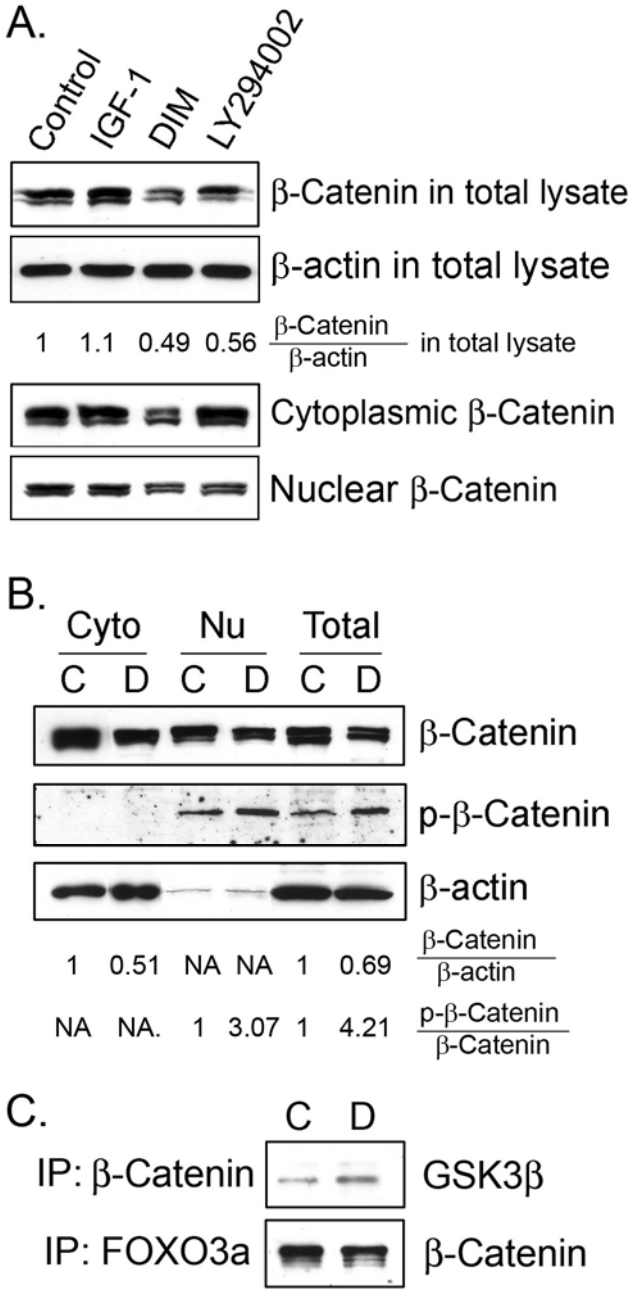

A.
Control
IGF-1
DIM
LY294002
β-Catenin in total lysate
β-actin in total lysate
1 1.1 0.49 0.56 β-Catenin / β-actin in total lysate
Cytoplasmic β-Catenin
Nuclear β-Catenin
B.
Cyto Nu Total
C D C D C D
β-Catenin
p-β-Catenin
β-actin
1 0.51 NA NA 1 0.69 β-Catenin / β-actin
NA NA. 1 3.07 1 4.21 p-β-Catenin / β-Catenin
C.
C D
IP: β-Catenin
GSK3β
IP: FOXO3a
β-Catenin

Figure 3

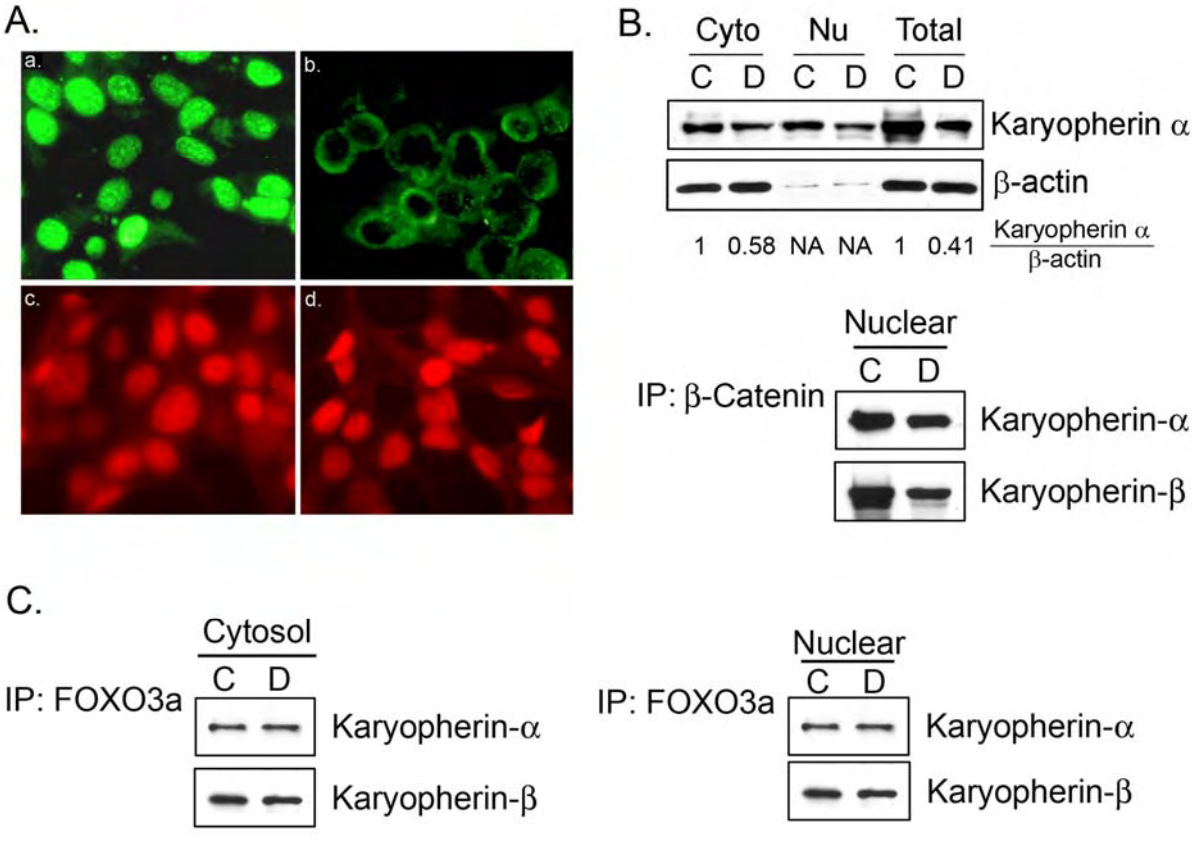

A.
a.
b.
c.
d.
B.
Cyto
Nu
Total
C D C D C D
Karyopherin α
β-actin
1 0.58 NA NA 1 0.41
Karyopherin α / β-actin
Nuclear
C D
IP: β-Catenin
Karyopherin-α
Karyopherin-β
C.
Cytosol
C D
IP: FOXO3a
Karyopherin-α
Karyopherin-β
Nuclear
C D
IP: FOXO3a
Karyopherin-α
Karyopherin-β

Figure 4

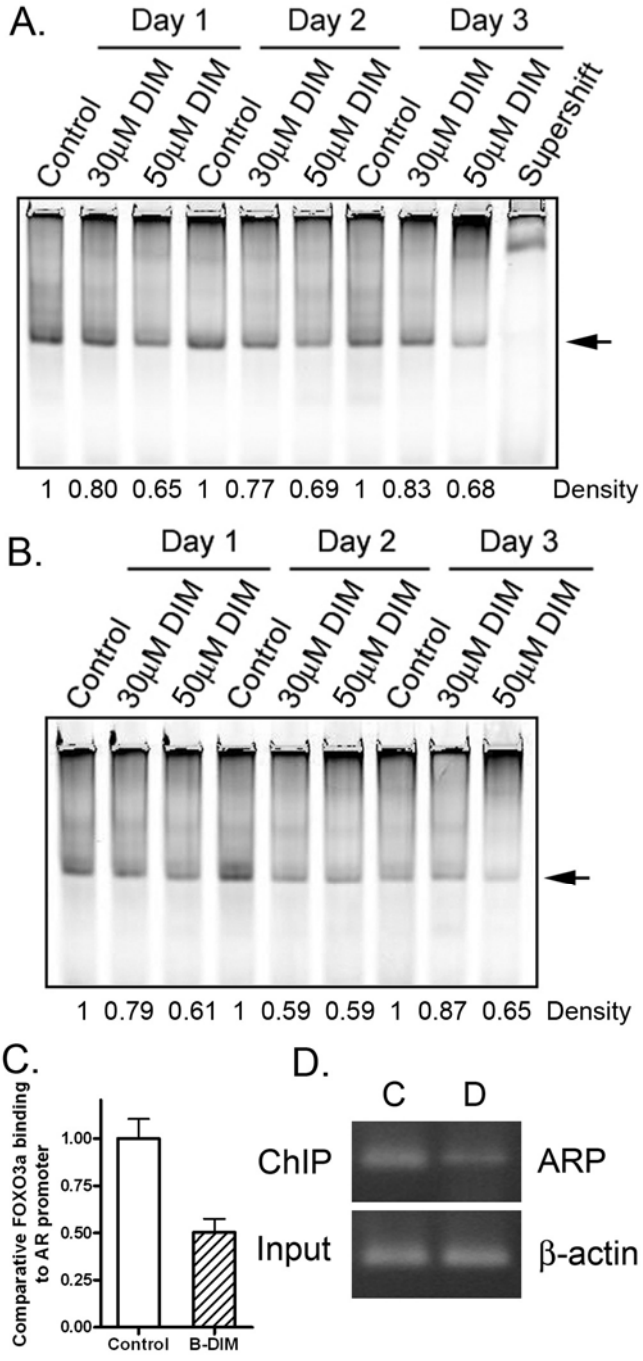

A.
Day 1
Day 2
Day 3
Control
30μM DIM
50μM DIM
Control
30μM DIM
50μM DIM
Control
30μM DIM
50μM DIM
Supershift
1 0.80 0.65 1 0.77 0.69 1 0.83 0.68 Density
B.
Day 1
Day 2
Day 3
Control
30μM DIM
50μM DIM
Control
30μM DIM
50μM DIM
Control
30μM DIM
50μM DIM
1 0.79 0.61 1 0.59 0.59 1 0.87 0.65 Density
C.
Comparative FOXO3a binding to AR promoter
1.00
0.75
0.50
0.25
0.00
Control
B-DIM
D.
C
D
ChIP
ARP
Input
β-actin

Figure 5

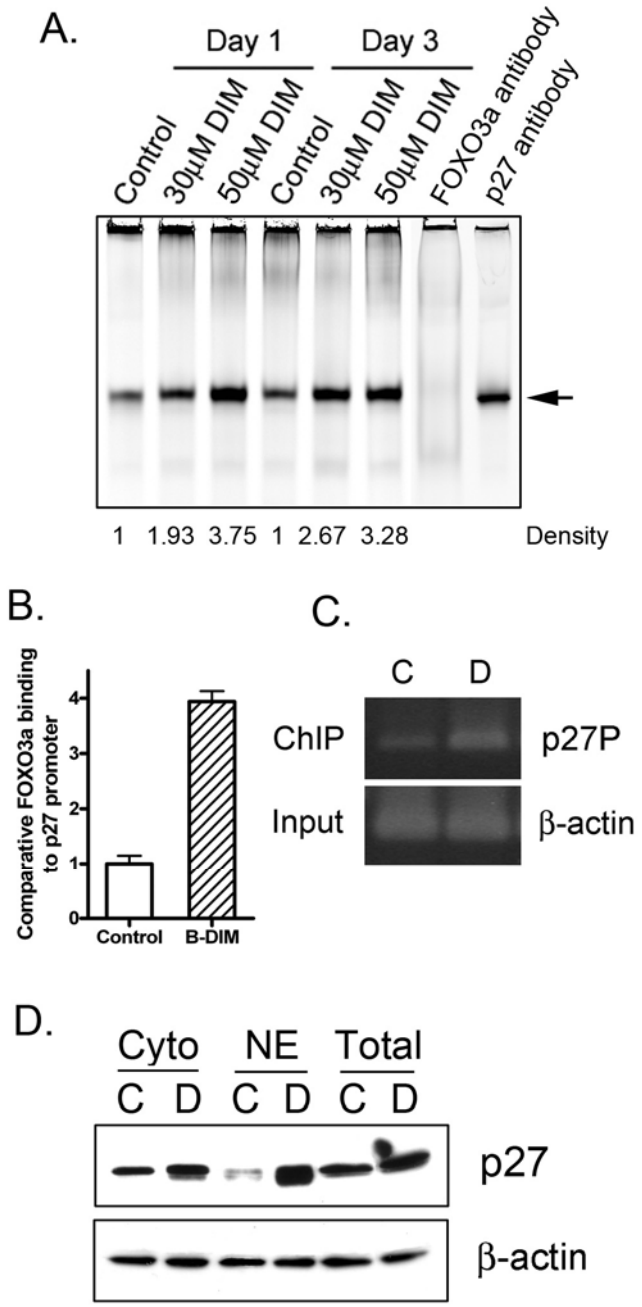

A.
Day 1
Day 3
Control
30μM DIM
50μM DIM
Control
30μM DIM
50μM DIM
FOXO3a antibody
p27 antibody
1 1.93 3.75 1 2.67 3.28
Density
B.
Comparative FOXO3a binding to p27 promoter
0
1
2
3
4
Control
B-DIM
C.
C D
ChIP
p27P
Input
β-actin
D.
Cyto NE Total
C D C D C D
p27
β-actin

Figure 6

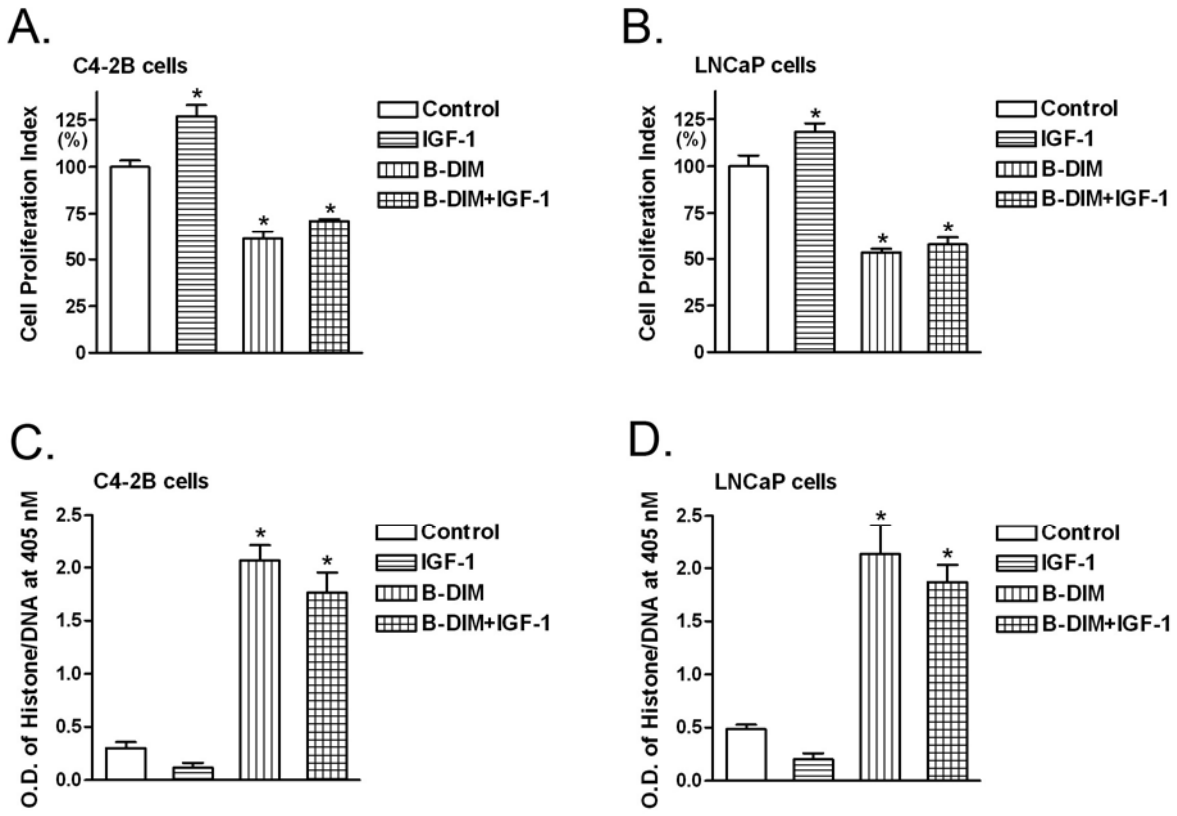
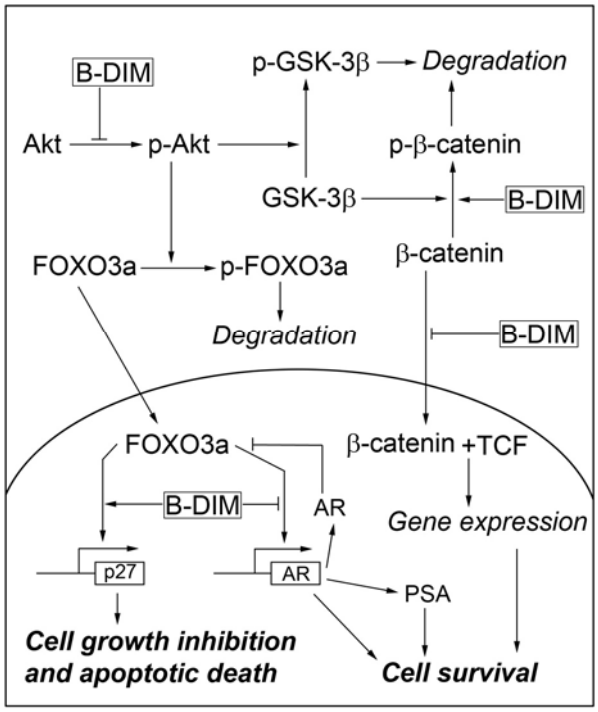

A.
C4-2B cells
Cell Proliferation Index
125
(%)
100
75
50
25
0
Control
IGF-1
B-DIM
B-DIM+IGF-1
B.
LNCaP cells
Cell Proliferation Index
125
(%)
100
75
50
25
0
Control
IGF-1
B-DIM
B-DIM+IGF-1
C.
C4-2B cells
O.D. of Histone/DNA at 405 nM
2.5
2.0
1.5
1.0
0.5
0.0
Control
IGF-1
B-DIM
B-DIM+IGF-1
D.
LNCaP cells
O.D. of Histone/DNA at 405 nM
2.5
2.0
1.5
1.0
0.5
0.0
Control
IGF-1
B-DIM
B-DIM+IGF-1

Figure 7


B-DIM
p-GSK-3β
Degradation
Akt
p-Akt
p-β-catenin
GSK-3β
B-DIM
β-catenin
FOXO3a
p-FOXO3a
Degradation
B-DIM
FOXO3a
β-catenin +TCF
B-DIM
AR
Gene expression
p27
AR
PSA
Cell growth inhibition
and apoptotic death
Cell survival

Appendix 14

Annual Meeting 2007
ABSTRACT SUBMITTER

Control/Tracking Number: 07-AB-1932-AACR
Activity: Abstract Submission
Current Date/Time: 11/27/2006 8:23:06 AM

Regulation of FOXO3a/β-Catenin/GSK-3β signaling by DIM contributes to inhibition of cell proliferation and induction of apoptosis in prostate cancer cells

Short Title:
DIM and FOXO3a/Catenin/GSK

Author Block: *Yiwei Li, Zhiwei Wang, Fazlul H. Sarkar.* Wayne State Univ. School of Medicine, Detroit, MI

Abstract:
Forkhead Box Class O (FOXO) transcription factor and glycogen synthase kinase 3 (GSK-3) are two targets of activated Akt and play important roles in cell cycle arrest and apoptotic cell death. β-Catenin has been found to interact with FOXO3a and GSK-3, and regulate their activities in the processes of cell proliferation and apoptosis. Moreover, FOXO3a, GSK-3, and β-Catenin all are androgen receptor (AR) coregulators and regulate the activity of AR, resulting in the development and progression of prostate cancer. Previous studies from our laboratory have shown anti-proliferative and pro-apoptotic effects of 3,3'-diindolylmethane (DIM) through regulation of Akt, NF-κB, and AR in prostate cancer cells. However, how DIM regulates Akt and AR singling pathways has not been fully investigated in AR positive hormone responsive and non-responsive prostate cancer cells. Here, we investigated the molecular effects of B-DIM, a formulated DIM with greater bioavailability, on Akt/FOXO3a/β-Catenin/GSK-3/AR signaling in hormone-sensitive LNCaP (AR+) and hormone-insensitive C4-2B (AR+) prostate cancer cells. We found that B-DIM significantly inhibited the phosphorylation of Akt and FOXO3a, and increased the phosphorylation of β-Catenin, leading to the inhibition of cell growth and the induction of apoptosis in both LNCaP and C4-2B prostate cancer cells. We also tested the interaction of these proteins in the cytoplasmic or nuclear compartment by immunoprecipitation. We found that B-DIM inhibited the formation of FOXO3a complex with Akt, AR, or β-Catenin. Moreover, B-DIM induced FOXO3a binding to GSK-3β and GSK-3β binding to β-Catenin. These results suggest that B-DIM could suppress Akt-induced FOXO3a phosphorylation and promote GSK-3β-induced β-Catenin phosphorylation. We also found that B-DIM significantly inhibited karyopherin, one of the nuclear importins, binding to β-Catenin, suggesting the inhibitory effect of B-DIM on β-Catenin nuclear translocation and subsequent AR transactivation. By EMSA and ChIP assay, we found that B-DIM inhibited FOXO3a binding to the promoter of AR and down-regulated AR transactivation, resulting in the inhibition of cell proliferation and the induction of apoptosis in androgen sensitive and insensitive prostate cancer cells. These results suggest that B-DIM induced cell growth inhibition and apoptosis induction are partly mediated through regulation of Akt/FOXO3a/β-Catenin/GSK-3β/AR signaling. From these results, we conclude that B-DIM could be a promising non-toxic agent for the prevention and/or treatment of both androgen-dependent and hormone-refractory prostate cancer.
:

Author Disclosure Block: **Y. Li**, None; **Z. Wang**, None; **F.H. Sarkar**, None.

Additional Disclosures (Complete):
The presenter of this abstract will discuss commercial products, devices, or technology in this presentation, as