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# Indole-3-carbinol mediated cell cycle arrest of LNCaP human prostate cancer cells requires the induced production of activated p53 tumor suppressor protein

*Jocelyn C. Hsu* [a], *Anurupa Dev* [a], *Aimee Wing* [a], *Christine T. Brew* [a], *Leonard F. Bjeldanes* [b], *Gary L. Firestone* [a,*]

[a] *Department of Molecular and Cell Biology and The Cancer Research Laboratory, The University of California at Berkeley, Berkeley, CA 94720, United States*

[b] *Department of Nutritional Sciences and Toxicology, The University of California at Berkeley, Berkeley, CA 94720, United States*

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*Abbreviations:*
I3C, indole-3-carbinol
DIM, 3,3′-diindolylmethane
TRYP, tryptophol
LNCaP, Lymph node carcinoma of the prostate
CDK, cyclin-dependent kinase
CKI, cyclin-dependent kinase inhibitor
ser, serine
siRNA, short interfering RNA
RNAi, RNA interference

ABSTRACT

Indole-3-carbinol (I3C), a dietary compound found naturally in cruciferous vegetables of the *Brassica* genus such as broccoli and brussels sprouts, induces a G1 growth arrest of human reproductive cancer cells. We previously reported that in LNCaP prostate cancer cells, I3C down-regulated cyclin-dependent kinase (CDK) 2 activity. In our current study, Western blotting and quantitative RT-PCR demonstrated that I3C treatment increased both the transcripts and protein levels of the CDK2 inhibitor $p21^{waf1/cip1}$ (p21). Transfection of luciferase reporter plasmids containing wild-type and mutated p21 promoter fragments revealed that I3C induced p21 gene transcription through a p53 DNA binding element. Oligonucleotide precipitation showed that I3C increased the level of activated p53 nuclear protein that is competent to bind its DNA target site on the p21 promoter. Ablation of p53 production using short interfering RNA (siRNA) prevented that the I3C induced G1 arrest and up-regulation of p21 expression. Western blots using p53 phospho-specific antibodies revealed that I3C treatment increased the levels of three phosphorylated forms of p53 (Ser15, Ser37, Ser392) that are known to contribute to p53 protein stability and greater transactivation potential. Taken together, our results establish that the I3C induced G1 arrest of human prostate cancer cells requires the induced production of the activated phosphorylated forms of p53, which stimulate transcription of the CDK2 inhibitor p21.

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* *Corresponding author at:* Department of Molecular and Cell Biology, 591 LSA, University of California at Berkeley, Berkeley, CA 94720-3200, United States. Tel.: +1 510 642 8319; fax: +1 510 643 6791.
E-mail address: glfire@berkeley.edu (G.L. Firestone).

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## 1. Introduction

Prostate cancer is the most prevalent cancer in men in the United States, and the second most common cause of cancer-related death in males [1]. Currently, the most common treatment for prostate cancer is androgen ablation therapy, which involves decreasing the amount of circulating androgens to inhibit cancer cell growth [2]. Given that prostate cancers initially develop as androgen dependent, removal of circulating androgens regresses the disease in 80% of patients [3]. However, patients inevitably progress to an androgen-independent state, thereby becoming resistant to the treatment [2]. Half of the patients who acquire resistance to androgen ablation therapy die within the first year following relapse, with the majority of the remainder succumbing to the disease within 2 years [1]. The lack of therapeutics effective against all types of prostate cancers remains a critical problem in confronting the disease.

One promising anti-cancer agent, indole-3-carbinol (I3C), a naturally occurring compound found in vegetables of the *Brassica* genus such as cabbage, broccoli, and brussels sprouts, has been shown to reduce tumor occurrences in mouse and rat models [4–8]. Exposure to dietary I3C reduces the incidence and multiplicity of mammary tumors while exhibiting negligible levels of toxicity [9]. When ingested, I3C is converted into a variety of acid-catalyzed derivatives that likely account for the activity of the dietary I3C pathway [10,11]. However, studies by us and others have shown that I3C itself has potent anti-proliferative activity when directly administered to cultured human breast and prostate cancer cells [12–15]. In addition, ectopic application of I3C directly inhibits skin tumor formation in mouse models [6]. I3C has been shown to induce a G1 cell cycle arrest of human reproductive cancer cells, although the precise molecular mechanism of I3C action remains elusive [12–15].

Cell proliferation is closely associated with regulated changes in the expression and/or activity of G1 cell cycle components. Key targets of these pathways are specific sets of cyclin/cyclin-dependent kinase (CDK) protein complexes, which function at the different stages of the cell cycle [16–18]. The appearance and activation of these cyclin-CDK complexes drives a cell from the G1 phase into the DNA synthesis S-phase of the cell cycle. In particular, the complexes of cyclin D/CDK4, cyclin D/CDK6, and cyclin E/CDK2 are especially important in the transition from G1 to S phase [16–18]. The activity of the CDK is tightly regulated by association with its cyclin, specific phosphorylation states, and possible interaction with a CDK inhibitor (CKI) [17,19–23]. One critical inhibitor of CDK activity is $p21^{waf1/cip1}$ (p21), which is known to bind to the cyclin-CDK complexes of CDK2, CDK4, and CDK6 following anti-mitogenic signals or DNA damage to inhibit their catalytic activity and induce cell cycle arrest. The importance of p21 in the regulation of the cell cycle has been established extensively and studies have shown that over-expression of p21 alone can cause a G1 cell cycle arrest [24]. Thus, p21 represents a potential target for therapeutics aimed against cancer cell proliferation.

We have previously demonstrated that I3C induced a G1 cell cycle arrest of LNCaP human prostate cancer cells [25]. This growth arrest was accompanied by inhibition of CDK2 and CDK4 activity without affecting the expression of their associated cyclins [25]. Non-transcriptional regulation of CDK activity often involves the presence or absence of a cyclin-dependent kinase inhibitor (CKI), such as p21. In order to understand the molecular mechanism behind the I3C induced growth arrest of prostate cancer cells, the early and sustained induction of p21 protein levels by I3C was characterized. We provide evidence that I3C up-regulates the expression of p21 through a p53-dependent mechanism that leads to a G1 arrest of LNCaP prostate cancer cells.

## 2. Materials and methods

### 2.1. Materials

The LNCaP human prostate carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA). RPMI Media 1640 with L-glutamine, HEPES buffer solution 1 M, and sodium pyruvate solution 100 mM, and lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Fetal Bovine Serum, calcium- and magnesium-free PBS and trypsin-EDTA were supplied by BioWhittaker (Walersville, MD). Dimethyl sulfoxide ($Me_2SO$, 99% high pressure liquid chromatography grade), I3C, DIM, and tryptophol were obtained from Aldrich (Milwaukee, WI). Forty percent of glucose solution was purchased from Sigma (St. Louis, MO). Agarose-conjugated oligonucleotides for the p53 consensus binding site and mutant p53 binding site as well as antibodies for p21, tubulin, and beta-actin were procured from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-hsp90 antibody from BD Biosciences (Palo Alto, CA). Antibodies for total p53 as well as phosphorylated forms of p53 (serine 4, 15, 20, 37, 46, 392) were purchased from Cell Signaling Technologies (Beverly, MA). Oligonucleotide primers for PCR were obtained from Integrated Diagnostic Technologies (Coralville, IA). Luciferase reporter constructs p21-luci and p21-DM-luci were generous gifts of Bert Vogelstein (Johns Hopkins University, MD). p21-Δp53-luci was created in our lab and described below. p53 validated siRNA was purchased from Ambion (Austin, TX) and negative control siRNA was a kind gift of Richard Stevens (University of California, Berkeley, CA)

### 2.2. Methods of culture

LNCaP cells were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 10% fetal bovine serum, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 1.25 mL of 20,000 units/mL penicillin/streptomycin. Cells were propagated in a 37 °C humidified chamber containing 5% $CO_2$. Cell culture medium was changed every 48 h. I3C, DIM, and tryptophol were dissolved in $Me_2SO$ at concentrations 1000-fold higher than the final concentration in medium. Final concentrations of treatments were 200 μM I3C, 50 μM DIM, and 200 μM tryptophol. These drug concentrations were determined by dose response treatments and chosen for maximal response with minimal cell death.

### 2.3. Western blot analysis

After the indicated treatments, cells were harvested in PBS and lysed in RIPA buffer (150 mM NaCl, 0.5% deoxycholate,

0.1% nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phophatase inhibitors (50 μg/mL PMSF, 10 μg/mL aprotinin, 5 μg/mL leupeptin, 0.1 μg/mL NaF, 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and 0.1 mM beta-glycerophosphate). Total cellular protein were quantified by the Bradford procedure and equal amounts of protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25 mL of 14.4 M 2-mercaptoethanol, 10% bromophenol blue, 3.13% stacking gel buffer) and fractionated by gel electrophoresis on 6%, 10%, or 12% polyacrylamide 0.1% SDS resolving gels. Rainbow marker (Amersham Pharmacia Biotech, Pistacaway, NJ) was used as the molecular weight standard. Proteins were transferred to nitrocellulose membranes (Micron Separations, Inc., Westborough, MA) and blocked overnight at 4 °C with 5% non-fat dry milk in 1× TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). Blots were subsequently incubated with primary antibodies in 1× TBST for 1 h. All p53 and phosphorylated p53 western blots were blocked for 1 h at room temperature in 5% non-fat dry milk in 1× TBST and then incubated with primary antibodies in 5% bovine serum albumin in 1× TBST overnight at 4 °C. Goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA) were used at 1:5000 dilution in 1× TBST with 1% non-fat dry milk. Blots were treated with Western Lightning Western Blot Chemiluminescence Reagent (Perkin-Elmer Life Sciences, Boston, MA) and the proteins were detected by autoradiography. Equal protein loading was ascertained by ponceau S staining of blotted membranes as well as Western blotting with beta-actin, Hsp 90 or tubulin antibodies.

### 2.4. Real-time quantitative and semi-quantitative RT-PCR analysis

After the indicated treatments, cells were rinsed with PBS and lysed with 1 mL Tri Reagent (Sigma). Total RNA was prepared according to the manufacturer's protocol, and treated with DNase I, amplification grade (Ambion) to remove DNA contamination. Two micrograms of total RNA was used to synthesize cDNA using Moloney murine leukaemia virus-reverse transcriptase (Promega, Madison, WI) with a random hexamer as a primer in a 20 μL reaction. Real-time quantitative PCR was performed using SYBR Green PCR Core Reagents and GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using universal cycling conditions. Amplification reactions contained 5 ng cDNA template, components of SYBR Green PCR Core Reagents according to the manufacturer protocol, and 300 nM of each primer in a final volume of 25 μL. The comparative threshold cycle ($C_T$) method was used to quantify data using b-actin as the normalizing gene. Semi-quantitative PCR was performed using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's conditions. Amplification reactions contained 200 ng cDNA template in a final volume of 50 μL. The PCR reaction was visualized by adding Blue/Orange 6× DNA loading dye (Promega, Madison, WI) and loading the reactions on a 1% agarose gel for electrophoresis. Human beta-actin real-time PCR primers were purchased from Biosource (Camarillo, CA). Primers used for amplification of p21 and p53 cDNA were carefully designed to span an intron to prevent amplification of genomic DNA and are as follows:

- p53 fwd: 5′-CTG TCC CTT CCC AGA AAA CC-3′;
- p53 rev: 5′-CCA CTC GGA TAA GAT GCT GA-3′;
- p21 fwd: 5′-CGA CTG TGA TGC GCT AAT GG-3′;
- p21 rev: 5′-CCA GTG GTG TCT CGG TGA CA-3′.

### 2.5. Transfection and luciferase assay

For reporter assays, transfections were performed by mixing 0.8 μg of reporter plasmid with 6 μL of enhancer and 20 μL of Effectene reagent in buffer EC according to manufacturer protocol (Qiagen, Valencia, CA). Reactions were quenched after 5 h by removal of transfection medium, replaced by treatment medium. All transfections were done in triplicate. For luciferase assays, cells were harvested by rinsing with PBS and lysed in 500 μL 1× passive lysis buffer (Promega) for 15 min with gentle rocking. Twenty microliters of cell lysate was added to 8 mm × 75 mm cuvettes (Promega) and subsequently loaded into a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA) after addition of 100 μL of Luciferase Assay Reagent II (Promega). Luminescence was measured in relative light units. The luciferase specific activity was expressed as an average of relative light units produced per μg of protein present in corresponding cell lysates as measured by the Bradford Assay (Bio-Rad).

### 2.6. Site-directed mutagenesis

A single base pair mutation was created in the p53 binding site of the p21 promoter-luciferase vector using the Site-directed Mutagenesis XL Kit from Qiagen. The primers used for construction of the mutant were:

- p53-mut-fwd: 5′-TCT GGC CAT TAG GAA AAT GTC CCA ACA TGT TGA G-3′;
- p53-mut-rev: 5′-CTC AAC ATG TTG GGA CAT TTT CCT AAT GGC CAG A-3′.

### 2.7. Preparation of nuclear extracts and oligonucleotide-precipitation

Preparation of nuclear extracts from was based on a method described previously. Briefly, cells were hypotonically lysed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF), allowed to swell for 30 min, gently homogenized in a dounce homogenizer, and centrifuged at 550 rpm to isolate nuclei. Nuclei were homogenized with a micro-pestle and then incubated in buffer C (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) for 30 min on a rocking platform to strip DNA-binding proteins. After centrifugation at 14,000 rpm, the resulting supernatant was dialyzed against buffer D (20 mM HEPES pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) overnight and then frozen at −80 °C. The protein content in the nuclear extracts was normalized by the Bradford procedure.

For the oligonucleotide-precipitation experiment, agarose-conjugated oligonucleotides were washed in 1× binding

buffer, which was diluted from its 3× buffer B (60 mM HEPES pH 7.9, 7.5 mM $MgCl_2$, 36% glycerol, 3 mM DTT, 3 mM EDTA, 180 mM KCl) and subsidized with 1 μg/mL of poly-dI/dC and 10 mM DTT. Fifty microliters of oligo-beads were used to pre-clear the nuclear extracts by incubating them with 200 μg nuclear extracts for 1 h at 4 °C on a rocking platform. Pre-cleared supernatant was transferred to new tubes and incubated with 50 μL of new oligo-beads, 500 μL of 1× binding buffer, and incubated on a rocking platform for 2 h at room temperature. Competitive control reactions were also incubated with 100-fold excess (75 μL of 10 μM annealed oligo stock) competitive oligonucleotides of the wild-type or mutant p53 binding site from the p21 promoter. The unbound proteins were collected and the protein-bound beads were washed with 1 mL 1× binding buffer three times with spins of 3 min at 13,000 × *g* in between washes. Spins were done at 4 °C. After the final wash, the protein-bound beads were incubated with 50 μL 1× binding buffer and 5× loading buffer from the western blot procedure and boiled for 5 min. Samples were then resolved on an SDS-PAGE gel according to the Western protocol described above by probing for p53 and hsp 90 antibodies. The competitive oligonucleotide sequences are as follows:

- p53-wt-fwd: 5′-TCT GGC CAT TAG GAA CAT GTC CCA ACA TGT TGA G-3′;
- p53-wt-rev: 5′-CTC AAC ATG TTG GGA CAT GTT CCT AAT GGC CAG A-3′;
- p53-mut-fwd: 5′-TCT GGC CAT TAG GAA AAT GTC CCA ACA TGT TGA G-3′;
- p53-mut-rev: 5′-CTC AAC ATG TTG GGA CAT TTT CCT AAT GGC CAG A-3′.

### 2.8. *Transfection of siRNA*

All siRNA was diluted to 50 μM stock concentration and used as a 1000× stock. Transfection of siRNA was done in six-well tissue culture plates with cells at 20% confluency. siRNA was introduced into the cells using lipofectamine 2000 transfection reagent according to manufacturer's protocols (Invitrogen). The cells were incubated in the transfection reagent and siRNA for 24 h and then subjected to a second round of transfection using the same procedure. After 24 h incubation with the second round of transfection, the cells were treated with DMSO or 100 μM I3C for 48 h. Cells were then harvested for flow cytometry or protein lysis for Western blotting to determine the effect on cell cycle and downstream targets.

## 3. Results

### 3.1. *I3C stimulates p21 protein and mRNA expression in LNCaP cells*

To determine the mechanism through which I3C causes a G1 cell cycle arrest of human LNCaP prostate cancer cells, production of the CDK inhibitor p21 was examined by Western blot analysis of cells treated with or without 200 μM I3C during a 48 h time course. The level of p21 protein was compared to that of β-actin protein. As shown in Fig. 1A, I3C treatment

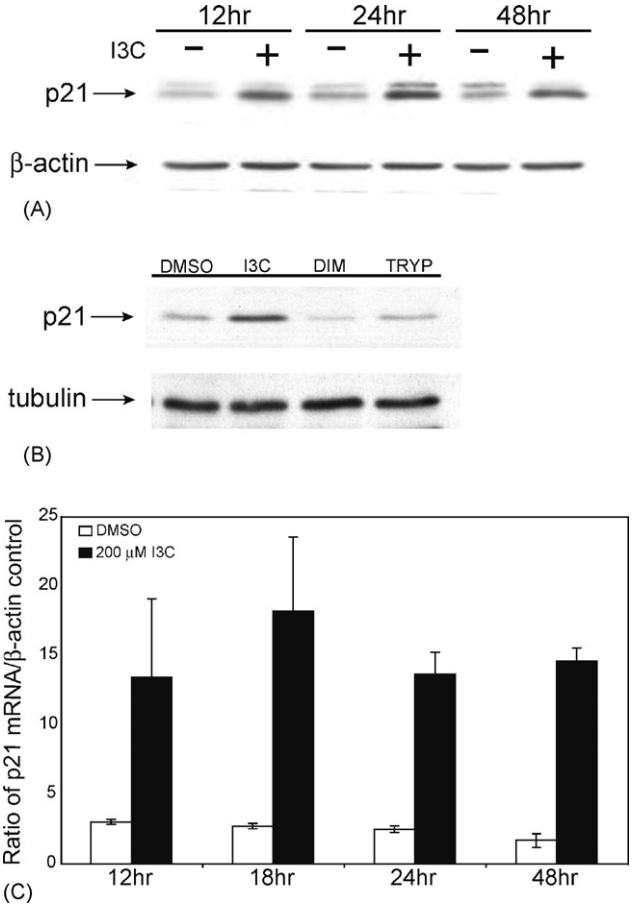


**Fig. 1 – I3C induces p21 protein and mRNA expression. (A) LNCaP cells were treated with DMSO vehicle control or 200 μM I3C for 12, 24, or 48 h, and a western blot of total cell extracts was probed for p21 protein production. Analysis of β-actin was used as a loading control. (B) LNCaP cells treated with 200 μM I3C, 50 μM DIM, 200 μM tryptophol, or without indoles (DMSO) for 48 h, and western blots used to analyze p21 protein production. Tubulin was used as a loading control. (C) To examine p21 mRNA expression, LNCaP cells were treated with or without 200 μM I3C for a 24 h time course, and total RNA was isolated, converted to cDNA, and the level of p21 transcripts quantified by real-time RT-PCR. β-Actin used as normalizing control and the graph represents the ratio of p21 mRNA/β-actin mRNA.**

strongly induced the production of p21 protein as early as 12 h of treatment, which was maintained through the 48 h time point. The rapid response of p21 expression regulation correlated strongly with the ability of I3C to inhibit the activity of CDK2 within 18 h of treatment and cause a G1 arrest within 24 h [25]. In indole treated breast cancer cells, a portion of I3C is converted into its natural dimerization product 3,3′-diindolylmethane (DIM), which also acts as a growth inhibitor [26–28]. In order to ascertain whether the up-regulation of p21 protein expression was an I3C specific response, LNCaP cells were treated with DIM or with tryptophol (TRYP), an indole compound with a structure that differs from I3C by one methyl

group and lacks an anti-proliferative response. Cells were treated for 24 h with each respective indole and p21 protein levels were determined by Western blot analysis. As shown in Fig. 1B, treatment with I3C, but not with tryptophol resulted in an increased level of p21 protein, showing that this response is not simply a nonspecific indole effect. Furthermore, the induction of p21 protein is not an indirect consequence of cell cycle arrest because DIM had no effect on p21 protein levels despite its ability to cause a G1 arrest in LNCaP cells [28].

To determine whether the increase in p21 protein levels resulted from transcriptional activation, p21 mRNA expression was measured by quantitative RT-PCR of total RNA isolated from I3C treated and untreated LNCaP cells. As shown in Fig. 1C, I3C induced the level of p21 transcripts as early as 12 h of treatment, and this response was maintained through 48 h. An increase in p21 mRNA levels was not observed at time points earlier than 12 h of I3C treatment (data not shown). The relatively rapid stimulation of p21 transcripts from I3C treatment corresponded with and likely accounted for the up-regulation of p21 protein.

### 3.2. I3C stimulation of p21 promoter activity requires the p53 DNA element

To determine whether I3C stimulates p21 promoter activity, cells were transiently transfected with a wild-type p21 promoter-luciferase reporter plasmid containing the −2.4 kb p21 promoter sequence (Fig. 2, left panel). Luciferase activity was monitered in transfected cells treated with or without I3C for 18 h because the maximal induction of p21 mRNA levels occurs by 18 h of I3C exposure. As shown in Fig. 2, I3C treatment resulted in an increased level of p21 promoter activity by approximately three-fold, which corresponds to the induction of p21 transcript and protein levels.

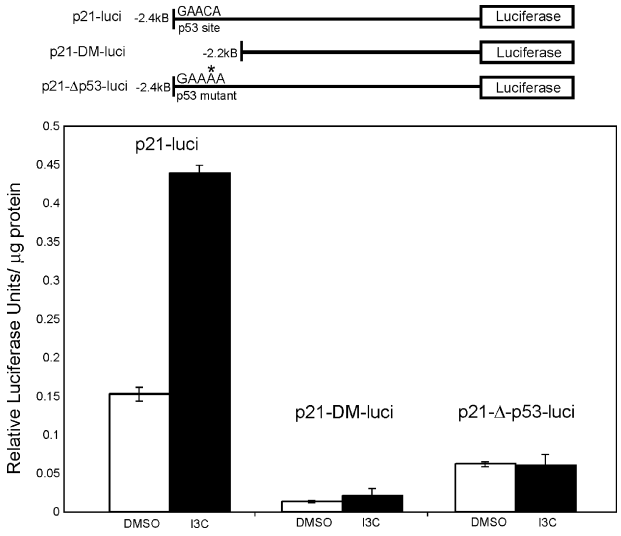


**Fig. 2 – I3C stimulation of p21 promoter activity requires the p53 DNA binding site. LNCaP cells were transfected with the wild-type p21 promoter-luciferase vector (p21-luci), or the p21 promoter with a 200 bp 5′ deletion (p21-DM-luci), or the mutant p21 promoter with a single bp mutation of the p53 binding site (p21-Δp53-luci). The cells were treated with DMSO control or 200 μM I3C for 24 h. The resulting extracts were incubated with luciferase substrate reagent and the relative light units of the enzymatic reaction quantified. The graph represents the relative light units normalized by protein concentration (RLU/μg protein).**

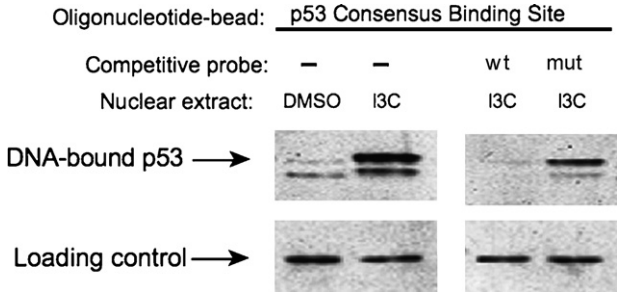


**Fig. 3 – Analysis of p53 DNA binding activity from I3C treated and untreated cells using an *in vitro* oligonucleotide precipitation assay. Nuclear extracts from DMSO (lane 1) or I3C (lane 2) treated cells were incubated with agarose-conjugated oligonucleotide beads containing the consensus p53 binding site. The reactions were subjected to Western blot analysis for DNA bound p53 protein that was eluted from the oligonucleotide-bead. To demonstrate specific p53 DNA binding capabilities to its target site on the p21 promoter, oligonucleotides corresponding to the wild-type (wt) (lane 3) or mutant (mut) (lane 4) p53 binding sites from the p21 promoter were incubated at 100-fold excess with the oligonucleotide beads containing the consequence p53 DNA binding site.**

To identify the DNA element required for I3C regulation of p21 promoter activity, luciferase reporter plasmids were constructed with a 5′ deletion and a site-directed point mutation on the p21 promoter. As shown in the promoter diagrams of Fig. 2, promoter constructs p21-luci, p21-DM-luci, and p21-Δp53-luci represent, respectively, luciferase reporter vectors driven by either the wild-type −2.4 kb p21 promoter, the −2.2 kb deletion construct of the p21 promoter, and the mutant −2.4 kb promoter with a single base pair mutation in the p53 binding site as generated by site-directed mutagenesis. LNCaP cells were transiently transfected with these reporter constructs and treated with or without I3C for 18 h. As shown in Fig. 2, I3C requires the presence of the wild-type p53 binding element on the p21 promoter in order to stimulate p21 promoter activity.

### 3.3. I3C induces an accumulation of activated p53 that binds to its p21 promoter element

An *in vitro* DNA-protein binding assay was utilized to test whether I3C induces the binding of p53 to the p21 promoter (Fig. 3). Agarose-conjugated oligonucleotides containing the consensus p53 DNA binding sites were incubated with nuclear extracts from either I3C treated or untreated LNCaP cells. After multiple washes, the oligonucleotide-bound proteins were eluted and electrophoretically fractionated. Western blots with an antibody specific for p53 showed that I3C treatment resulted in a significant increase in the level of

p53 protein able to bind to its consensus DNA binding site (Fig. 3, left panel). Incubation with excess oligonucleotide corresponding to the p53 DNA element within the p21 promoter quantitatively competed with the bead-bound p53 consensus DNA element for p53 binding (Fig. 3, wt competitive probe). In contrast, incubation of the reaction mixture with excesss oligonucleotide containing the mutated p53 DNA element of the p21 promoter failed to compete with the consensus DNA element for p53 binding (Fig. 3, mut competitive probe). Thus, I3C treatment induces activated p53 that binds to its target DNA element on the p21 promoter.

### 3.4. I3C up-regulates p53 protein levels without altering p53 transcript levels

One mechanism by which I3C could stimulate the level of DNA bound p53 is to induce total p53 protein levels. Western blot analysis of cells treated with either 100 μM or 200 μM I3C for either 12 h or 24 h showed that I3C treatment strongly up-regulated the production of total p53 protein (Fig. 4A) as compared to DMSO treated cells. The I3C mediated induction of p21 protein levels, a p53 target gene, corresponded with that of p53 protein. Semi-quantitative RT-PCR of total mRNA extracted from LNCaP cells treated with or without I3C resulted showed that I3C had no effect on p53 transcript levels during the first 12 h of indole treatment (Fig. 4B upper panels). I3C induced p53 protein levels at 3 h, 6 h and 12 h of indole treatment (Fig. 4B, lower two panels) under conditions in which there were no effects on p53 transcript levels (Fig. 4B, upper two panels). Thus, I3C induces the rapid stimulation of p53 protein levels by regulating posttranscriptional cellular processes.

### 3.5. I3C induces specific phosphorylated forms of p53

Previous studies have shown that phosphorylation of p53 controls protein stability and transactivation potential (29). To determine whether I3C treatment regulates the state of p53 phosphorylation, LNCaP cells were treated for 12 h or 24 h with or without I3C, and Western blotted with a series of phosho-p53 specific antibodies. As shown in Fig. 5, I3C stimulated the level of p53 phosphorylated at Ser15, Ser37 and Ser392, whereas, I3C had no effect on p53 phosphorylation at Ser4, Ser20 or Ser46. The Ser6 and Ser9 phosphorylated forms of p52 were not detected in the absence or presence of I3C (data not shown). Of the three I3C induced forms of phospho-p53, phosphorylation of Ser15 and Ser37 are known to stabilize p53 protein and prevent its degradation [29], whereas, phosphorylation at Ser392 has been shown to be essential for p53 binding to its DNA element [30–33].

### 3.6. Ablation of p53 protein production by short interfering RNA (siRNA) prevents the I3C induced G1 cell cycle arrest and up-regulation of p21 expression

To determine whether the cell cycle arrest caused by I3C treatment requires the induction of p53 protein, RNA interference (RNAi) specific for p53 was transfected into LNCaP

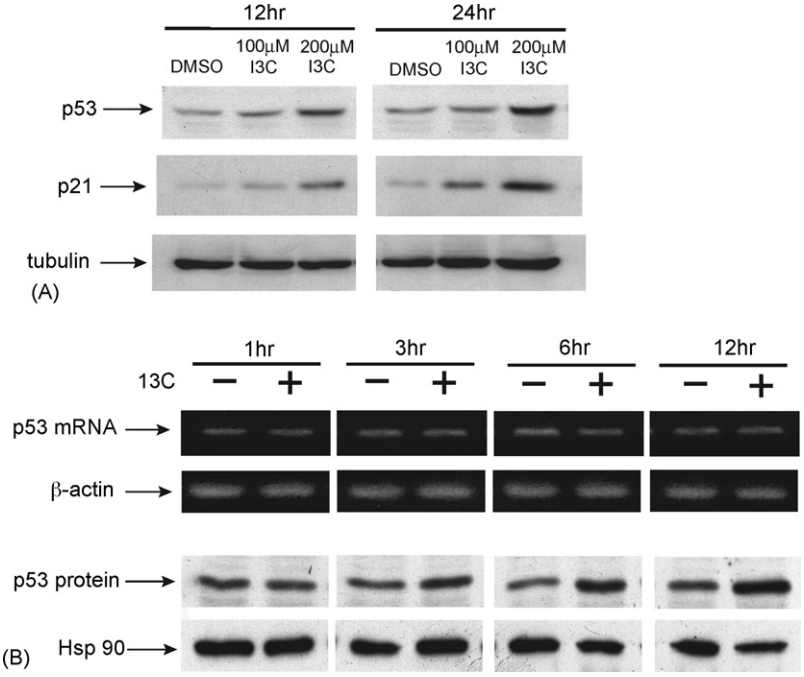


**Fig. 4 – I3C up-regulates the levels of p53 protein but does not regulate p53 transcript levels. (A) LNCaP cells were treated for 12 h or 24 h with DMSO, 100 μM I3C, or 200 μM I3C. The levels of p53 and p21 protein, as well as a β-actin control, were examined by western blot analysis. (B) LNCaP cells were treated with or without 200 μM I3C for the indicated time points and total RNA or cell extracts isolated as described in the text. p53 transcript levels were examined by semi-quantitative RT-PCR (top panel) and p53 protein levels determined by western blots (bottom panel). β-Actin was used as a loading control for RT-PCR, whereas, heat shock protein 90 (hsp 90) was used as a loading control for the Western blot.**

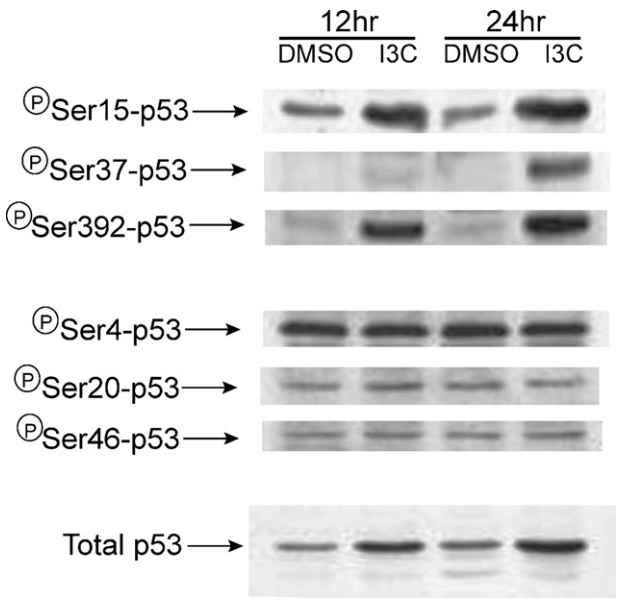


**Fig. 5 – I3C induces the level of specific phosphorylated forms of p53 protein. LNCaP cells were treated for 12 h or 24 h with DMSO control or 200 μM I3C and the indicated phosphorylated forms of p53 examined by western blots using individual phospho-specific p53 antibodies. The top panel shows the phospho-p53 forms that are regulated by I3C, the middle panel shows phospho-p53 forms that are not under indole control, and the bottom panel shows the production of total p53 protein.**

cells, which were then treated with or without I3C. The cells were tested for the I3C induction of p53 protein by Western blot and for cell cycle arrest by flow cytometry. As shown in Fig. 6, transfection of the p53 siRNA prevented the I3C induction of p53 and p21 protein levels (Fig. 6A, right panels), whereas, transfection of a control siRNA, had no effect on the I3C induction of either p53 or p21 protein (Fig. 6A, left panels). Expression of p53-specific or control siRNA had no effect on the I3C down-regulation of androgen responsive prostate specific antigen expression (data not shown) suggesting that p53 independent responses to this indole are unaffected by a disruption in p53 production. The p53 specific siRNA abrogated production of p53 in both the I3C treated and untreated cells, demonstrating the efficiency of this reagent.

Flow cytometry of propidium iodide stained nuclei from LNCaP cells treated with or without I3C revealed that transfection of p53 siRNA prevented the I3C mediated G1 cell cycle arrest. In cells transfected with the control siRNA, I3C stimulated the number of cells arrested in G1 phase and significantly reduced the percentage of S phase cells (Fig. 6B, right panels). Transfection of p53 siRNA abrogated the ablated the I3C induction of cG1 cell accumulation and down-regulation of S phase cells. This result demonstrates that the production of p53 is required for I3C mediated cell cycle arrest, and is the first documentation that ablation of expression of one I3C inducible gene product is sufficient to interfere with the I3C growth arrest of a human reproductive cancer cells.

## 4. Discussion

Relatively little is known about the anti-proliferative mechanisms of dietary indoles in human prostate cancer cells. The natural indole I3C induces a G1 cell cycle arrest of both androgen responsive and nonresponsive human prostate cancer cells [25]. Our current results have demonstrated a direct link between regulation of the p53 tumor suppressor protein and I3C inhibition of LNCaP cell growth. I3C treatment stimulated the level of p53 protein, which was responsible for induction of p21 expression at the transcriptional level. Under conditions in which I3C treatment resulted in elevated p53 protein levels, this indole had no effect on p53 transcripts, suggesting post-translational regulation of p53. The induction of specific phosphorylated forms of p53 by I3C treatment likely accounts for its stabilization and activation. The up-regulation of p53 proved to be an essential mechanism in the I3C anti-proliferative pathway because transfection of cells with p53 siRNA prevented the I3C mediated G1 cell cycle arrest and inhibited I3C induction of p21 gene expression. Thus, I3C requires activated p53 protein to carry out its growth arrest function. Taken together, our results demonstrate for the first time that the disrupted production of one I3C inducible protein, p53, can ablate the indole mediated cell cycle arrest.

The p53 tumor suppressor protein is highly regulated by post-translational modifications such as phosphorylation and acetylation. Western blots using phospho-p53 antibodies revealed that the I3C inducible p53 protein is phosphorylated at the Ser15, Ser37, and Ser392 residuces, which are sites known to be important for p53 protein stability and transcriptional activity [29,32,34–42]. Phosphorylation at the N-terminus of p53 such as Ser15 and Ser37 typically is associated with protein stability through the disruption of p53-MDM2 interaction [43,44]. The dissociation of MDM2 causes inhibition of p53 ubiquitination since MDM2 is the ubiquitin ligase for p53 protein, leading to an accumulation of p53 in the cell. However, in order to induce p53 transactivation capabilities, phosphorylation of p53 at Ser392 is considered necessary [29]. The Ser15 residue in p53 has been shown to be phosphorylated by ATM, ATR, DNAPK, ERK1/2, or p38 MAPK, all of which respond to DNA damage or UV light [29,45–47]. ATR and DNAPK can also phosphorylate serine 37 [29]. Ser392 phosphorylation occurs upon activation of p38 MAPK as well as FACT-CK2 and PKR, the latter being activated by interferon and the former two by UV light [29]. However, it is still unknown how treatment of a dietary indole could activate p53 phosphorylation in the absence of DNA damage or UV light. Initial experiments with inhibitors of the upstream kinases that target p53 at the I3C-activated sites have not uncovered a potential candidate kinase that is regulated by I3C. I3C could regulate the p53 phosphorylation states by stimulating the activity or expression of a protein kinase or by inhibiting a phosphastase that targets p53. Our future experiments are aimed at identifying the I3C regulated signaling component that controls p53 phosphorylation in order to elucidate I3C signaling components that regulate of prostate cancer cell growth.

The p21 CDK inhibitor is a transcriptional target gene of p53, and consistent with the I3C induction of p53 protein levels, I3C stimulated transcription of the p21 gene through a

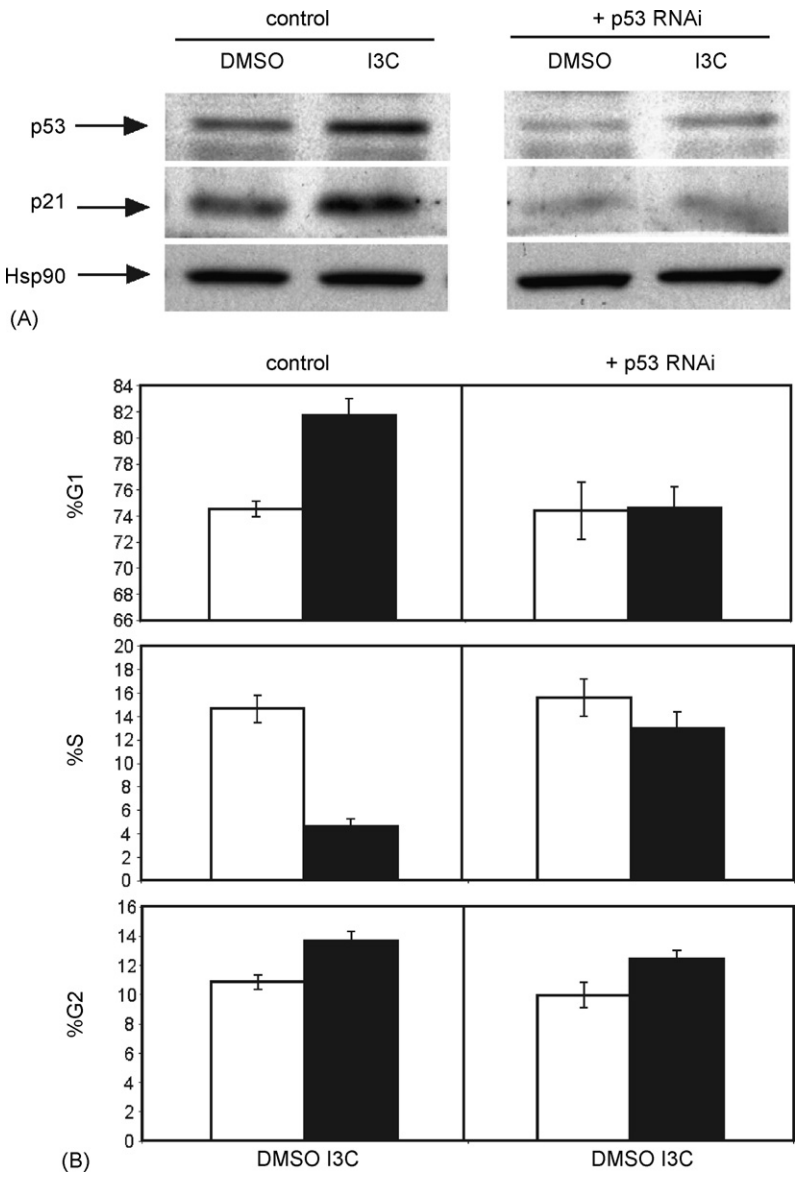


**Fig. 6 – Ablation of p53 production by p53-specific interfering RNA prevents the I3C induction of p21 and abrogates the I3C-induced G1 cell cycle arrest. (A) Control siRNA (left panel) or p53 siRNA (right panel) was transfected into LNCaP cells prior to treatment of DMSO or 100 μM I3C. The control siRNA is a random siRNA that is not targeted towards any particular gene. LNCaP cells were transfected twice for 24 h and then treated with indole for 48 h before harvesting for Western blot assay of p53 and p21 protein levels. Hsp 90 was used as loading control. (B) LNCaP cells were transfected with p53 siRNA (right panel) or control siRNA (left panel), and treated with DMSO or 100 μM I3C for 48 h. Harvested cells were stained for DNA using propidium iodide and DNA content analyzed by flow cytometry. The percentage of cells in G1, S, or G2 phase are shown to demonstrate the difference in cell cycle distribution between DMSO or I3C treated cells transfected with either control siRNA, or p53 specific siRNA.**

p53-dependent mechanism. The induction of p21 promoter activity required the p53 DNA element, and ablation of p53 expression using RNAi prevented the I3C induction of p21 gene expression in LNCaP prostate cancer cells. The I3C regulation of p21 expression accounts for the decrease in CDK2 kinase activity that we previously reported [25]. Thus, in LNCaP prostate cancer cells, I3C mediates a G1 cell cycle arrest by stimulating p53 protein levels and inducing p21 gene transcription. It is important to note that in breast cancer cells that express a mutant form of p53, the I3C mediated cell cycle arrest is not dependent on p53 production or increased expression of the p21 CDK inhibitor [12,13,15]. Therefore, the I3C signaling pathways may differ depending on whether a particular reproductive cancer cell types contains mutated p53 or wild-type p53.

Mutations in the p53 gene are generally believed to be a late event in the progression of prostate cancer, and are associated with androgen-independence, metastasis, and a poor prognosis [48]. According to most studies, p53 mutations are found in about 20–40% of cases in metastatic and/or hormone-refractory

prostate carcinomas. Wild-type p53 acts as an inducible transcription factor with multiple anti-proliferative roles in response to genotoxic damage [49,50], which can control the cell cycle transition from the G1 phase to the S phase. Under conditions conducive to DNA damage, p53 can either induce apoptosis or arrest the cell cycle for DNA repair [50]. Due to its role in cell cycle control and tumor progression, pharmacological strategies that control p53 functions could be potential candidates for prostate cancer therapy, used either alone or in combination with genetic strategies that help restore wild-type p53 in mutated cancer cell types. Our results, in combination with our previous studies showing that the effective intracellular concentration of I3C in human reproductive cancer cells is significantly lower than the level added to the cell culture medium [26], implicate I3C as a potential therapeutic agent for use in human prostate carcinomas due to the ability of this natural indole to induce a p53-mediated cell cycle arrest of cultured human prostate cancer cells.

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