Examining the Effects of Indole-3-Carbinol on Molecularly Different Osteosarcoma Cell Lines

By
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April 4, 2017



Allegheny College
Biochemistry Program

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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April 4, 2017

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Abstract
Indole-3-carbinol (I3C) is a phytochemical found in plants of the *Brassica* genus, which has been documented to stabilize a number of cancer sustaining pathways, including the G_1/S cell cycle transition. It was hypothesized that I3C decreases cancer proliferation at this checkpoint through the upregulation of tumor suppressor protein p21, and the subsequent inhibition of the retinoblastoma protein (Rb). To find a better understanding of the molecular consequences that mutations have on the efficacy of chemical treatments, this study observed how I3C functioned in canine osteosarcoma cell lines OSCA 32 and OSCA 40. These cell lines are distinguished by their relative activity of Rb, the key regulator in the G_1/S checkpoint. It was hypothesized that I3C would have little effect on the proliferation of OSCA 40 due to the abnormally low levels of Rb expression. The I3C lowered the cell proliferation in both OSCA 40 and OSCA 32, as measured using a cell proliferation assay. Reverse transcription and quantitative PCR was used and it was determined that there was no change in Rb or p21 mRNA levels in either cell line. This suggests I3C functions in other capacities to decrease cell proliferation in both cell lines.

Introduction

Osteosarcoma is the most commonly occurring form of bone cancer in children and teenagers. This cancer is named after the abnormal formations of the webbed protein osteoid in non-mineralized bone that causes the malignant cancer. Despite years of effort on the part of medical researchers, osteosarcoma continues to be debilitating and deadly for afflicted patients (Gill, et al., 2013). Research efforts are continuing to expand our knowledge of tumorigenic pathways and the growth of cancer cells, but cells are complex environments with endlessly branching pathways. Eukaryotic cells perform all of the basic functions that provide us with energy, detoxify harmful chemicals, repair afflicted damage, and produce more cells for growth and repair. All of these intricate processes provide an interminable list of potential modifications a cell can go through during a normal life cycle, and the buildup of harmful modifications over time can potentially lead to downstream effects on normal cellular proliferation. Once these cells start to proliferate without discrimination, they form malignant cancerous tumors and begin to stress the cells around them, preventing the tissue around it from functioning normally (National Cancer Institute, 2015). Further understanding, and potential treatment of certain cancers may be possible with a specific knowledge of the molecular abnormalities in these cells. We can narrow the endless possibilities of this search by looking at one very common process of tumorigenesis first, the inhibition of the cell cycle checkpoints via chemotherapeutic techniques.

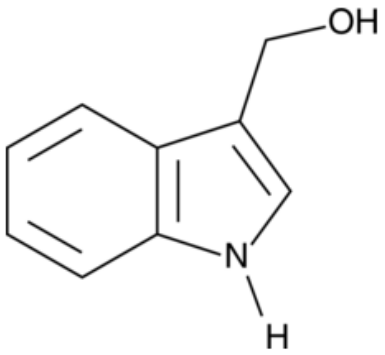

Figure 1. Indole-3-Carbinol
Adapted from Grose and Bjeldanes (1992).

There are wide range of cancer treatments utilized today, some work by returning cells to a non-dividing state. Chemotherapy uses cancer targeting drugs to prevent or slow aberrant cell division and induce apoptosis in the existing abnormal cells. However, major side effects of these drugs can be nausea, hair loss, and a general lower quality of life (Iwamoto, 2013). Looking into more traditional edible plant remedies could yield a treatment with fewer side effects and similar results. These "super foods" include cruciferous vegetables such as kale, broccoli, and collard greens. Some of the research into alternative treatments has focused on indole-3-carbinol (I3C), a byproduct of glucosinolate breakdown in these plants (Chinni et al., 2001). In addition to halting the growth of abnormally dividing cells, low toxicity treatments such as I3C have a selective effect, limiting the damage of the normally functioning cells around the cancer. This important observation contributes to why this drug is a promising alternative to the nondiscriminatory chemotherapy and radiation therapy currently in use, as it can provide treatment without many of the terrible side effects associated with other chemotherapeutics (Howells et al., 2002). While research on the efficacy of I3C has initially been focused on cancers dependent upon estrogen, newer studies are now being conducted on cancers that are not

influenced by estrogen. These studies include cells from prostate, liver, and lung cancers (Chinni et al., 2001; Howells et al., 2002; Choi et al., 2010; De Santi et al., 2011; Song et al., 2015; Wang et al., 2015). In these estrogen independent studies, I3C has been suggested to affect several pathways of tumorigenesis, including apoptosis and cell cycle arrest at the G_1/S phase checkpoint (Chinni et al., 2001; Zhang et al., 2003).

The cell cycle is a process of organismal growth and repair, multiplying healthy cell numbers when needed. The cycle begins with the quiescent G_0 phase, in an adult organism the majority of cells are in this phase (Figure 2). It is also the phase where everyday cell processes are carried out. The cell may be signaled out of the quiescent phase when the organism is growing, repairing a wound, or replacing naturally dead cells. The transition into the G_1 phase is marked by the cell expanding and synthesizing the mRNA, proteins, and organelles required for S phase DNA replication (Vermeulen et al., 2003; Giacinti and Giordano, 2006).

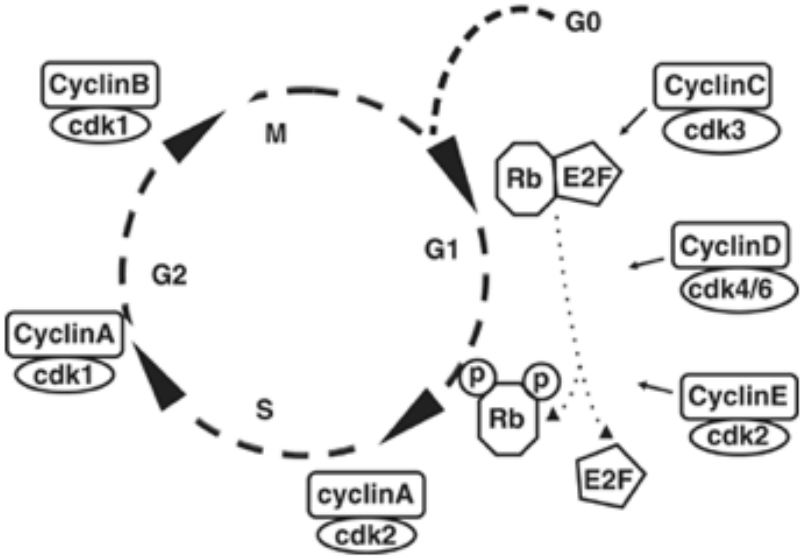

Figure 2. The Cell Cycle and G1/S checkpoint.
Adapted from Giacinti and Giordano (2006).

Before the S phase can begin, the cell must pass through the first regulation checkpoint, and cannot do so unless no structural DNA damage has occurred in the cell. This also ensures that the organism has enough resources, such as cytoplasm and nucleic acid building blocks, to support this growth (Vermeulen et al., 2003). This checkpoint begins primarily with the formation of three cyclin dependent kinase (CDK) complexes, cyclin-D/CDK4, cyclin-E/CDK2 and cyclin-D/CDK6. Kinases are enzymes used in many activation events in cell processes, they attach a phosphate group to the hydroxyl group of a specific serine, threonine, or tyrosine residue, depending on the target protein. This phosphate attachment is a change in the protein structure and therefore causes a change in the function of the protein. The CDK family contains an allosteric site where the cyclins bind. When cyclin is bound, the CDKs re-form their tertiary structure and open the enzyme active site, the site that facilitates protein phosphorylation. Therefore, kinases are dependent on cyclins to be actively functioning in cell signaling processes, as suggested by their name. The cell cycle utilizes these CDK/cyclin complexes as regulators in most phase checkpoints. Cyclin-D/CDK4, cyclin-E/CDK2, and cyclin- D/CDK6 complexes phosphorylate their respective retinoblastoma (Rb) protein complex targets to transition the cell into the S phase (Giacinti and Giordano, 2006; Lim and Kaldis, 2013). At the beginning of G_1, Rb is bound to the transcription factor E2F which blocks it from binding to its target genes, thus preventing the transcription of DNA replication proteins necessary for the S phase, such as DNA polymerase (Huang et al., 2001; Vermeulen, et al., 2003). However, when the Rb proteins are phosphorylated, their active site is altered causing the release of the bound E2F. The release of E2F then allows the cell cycle to continue (Giacinti and Giordano, 2006).

DNA damage is a general term that encompasses a multitude of DNA changes that affect the function of the DNA. Damage is detected by lesion specific repair factor proteins that scan

the DNA for defects such as double strand breaks, pyrimidine dimerization due to UV light, or base pair loss due to DNA hydrolysis (Rouse and Jackson, 2002). If DNA damage goes undetected, any changes to the DNA can be solidified as permanent mutations in subsequent cell cycle stages. These mutations can then lead to abnormal cell proliferation followed by potential cancerous effects. To help prevent this, tumor suppressor proteins are signaled, by the proteins scanning the DNA for defects, to activate downstream cell cycle arrest pathways. The tumor suppressor protein category includes proteins that directly cause cell cycle arrest, assist with DNA repair, induce apoptosis in cells with critical levels of DNA damage, or act as transcription factors for other proteins involved in cell proliferation (Hyland, n.d.).

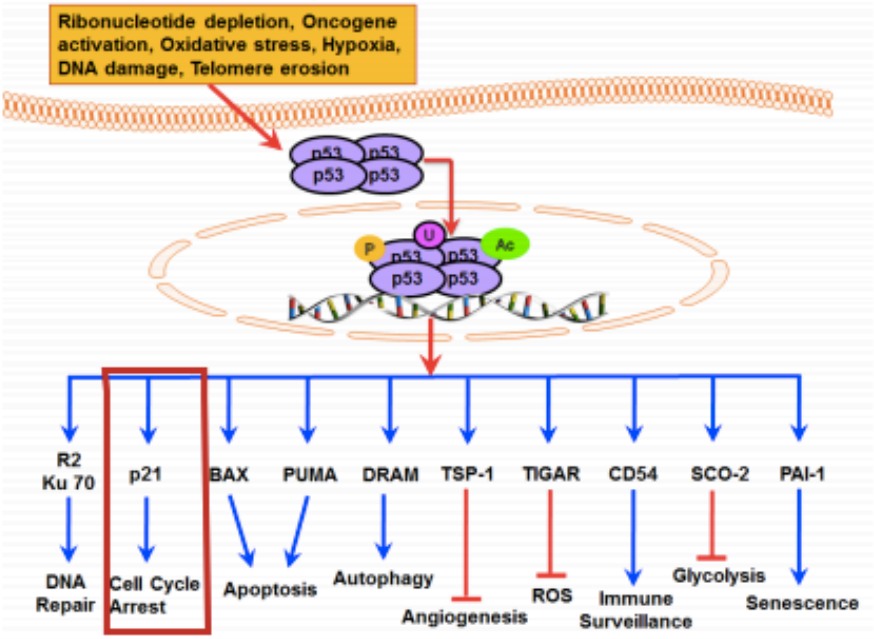

Figure 3. p53 binding targets. The top shows the p53 complex moving from the cytoplasm to the nucleus and binding to DNA due to a cell damaging event. Below are the proteins of the genes p53 activates. These proteins then affect a regulatory pathway to counteract the event. The arrows represent an activation event, and the T-Bars represent repression events. p53 binding to CDKNA1 DNA promotes p21 protein expression, subsequently activating cell cycle arrest. Adapted from Nag et al. (2013).

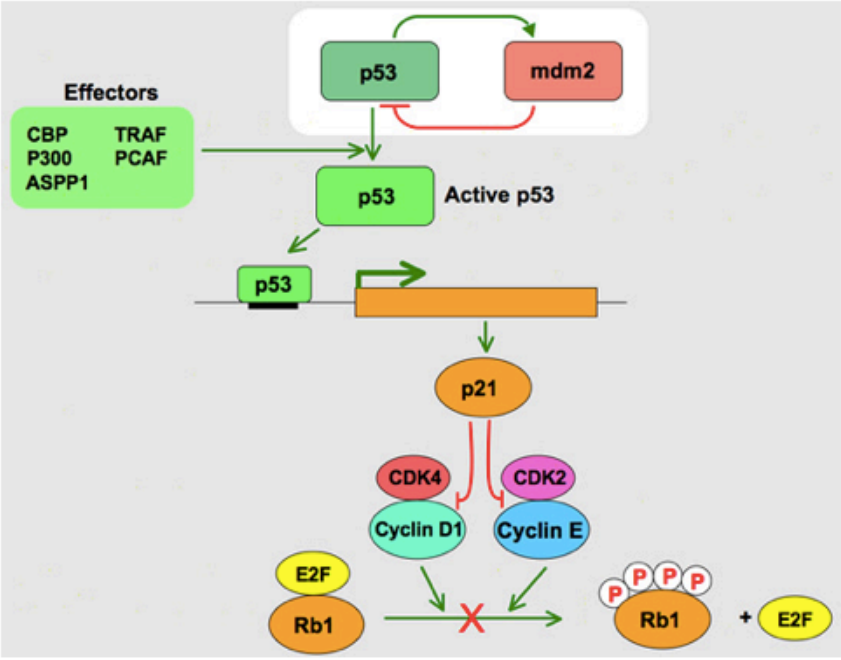

Figure 4. p53 regulation and the G_1/S cell cycle arrest. A further look into p53 downstream effects. The X on the bottom arrow represents p21 repressing CDK D1 and E therefore blocking the phosphorylation of Rb as depicted by the arrow. Rb is phosphorylated when p21 is activated. Adapted from Soussi (2012).

For the G_1/S phase, the major tumor suppressor protein is p53, which binds to the promotor region of multiple DNA targets (Figure 3). The p53 target proteins are responsible for maintaining the health of a cell as well as regulating its growth and death. Most importantly for this study, p53 binds to the cyclin dependent kinase inhibitor 1A gene (CDKN1A) when its inhibitor Mdm2 is not present (Brooks and Gu, 2010). As shown in Figure 4, p53 promotes the transcription of the p21 protein by binding to the CDKN1A gene. The p21 protein binds to the cyclin/CDK complexes formed in the G_1 phase and deactivates them. By inhibiting CDK phosphorylation, Rb remains bound to E2F. Therefore, as shown in Figure 3, p53 activation can halt the cell cycle at the G_1/S checkpoint through the Rb/E2F regulatory pathway (Löhr et al., 2003). However, damage to one of the many regulatory genes in this pathway can cause the

checkpoint to be nonfunctional and aberrant tumor cell division can occur.

One example of this type of tumorigenesis is a common form of retinoblastoma which contains a mutation in the Rb gene that renders it incapable of producing the protein. If there is no Rb to prevent E2F from promoting the continuation of the cell cycle, then the damaged cells could continue dividing indefinitely. This mutation would render the cell incapable of halting the cell cycle in response to p53 and p21 since the downstream target of of these proteins is missing (Giacinti and Giordano, 2006). Another possible tumor causing mutation in the Rb pathway is in the CDK genes. The mutation specifically causes constitutive phosphorylation of Rb, therefore rendering the checkpoint irreversibly deactivated, and the cell cycle constantly turned on. Other effects to this pathway have been studied in cancers, in fact the p53/Rb cascade has been mutated in some way in 80% of studied human cancers. If the Rb pathway and the rest of the regulatory G_1/S CDK checkpoints are negatively affected by a mutation in the cell, the abnormal functions can increase production of tumorigenesis through increased production of abnormal cells (Ortega, et al., 2002). Since Rb inactivation is such a common indicator of tumorigenic cells, many scientists have been focusing on chemotherapeutic techniques solely for the regulation of the Rb controlled G_1/S checkpoint. I3C is one of many chemicals shown to activate p53. It has also been shown to deactivate CDK formation by activating the inhibitors of CDK6 expression, such as p21 (Cover et al., 1998; Chinni et al., 2001; Choi et al., 2010).

Since the hypothesized target of I3C is a part of the G_1/S checkpoint, it may be of interest to observe the efficacy of I3C on multiple cancers of the same tissue with different molecular profiles centering around Rb functionality. These molecular profiles are due to the differing mutations a cell can go through to cause tumorigenesis. By comparing the same trials on molecularly unique cells, it may be found that chemical treatments need to be adjusted to

compensate for the disruption these molecular differences have on the pathways the treatment affects. For example, the cell lines used in this study are two phenotypically different osteosarcoma cell lines, denoted OSCA 40 and OSCA 32. The metastatic potential of these cell lines has been investigated by the use of xenotransplants in immune compromised mice, determining an association of OSCA 32 cells with longer patient survival rates and less aggressive cellular proliferation than the OSCA 40 line. The differences in the rate of cell growth for the two lines is due in part to their differences in gene expression (Scott et al., 2011; Scott et al., 2015). The most relevant difference in gene expression between the cell lines was observed to be that of the Rb1 gene. As depicted in Figure 5, OSCA 40 cells lack Rb protein expression while the OSCA 32 cells have high levels of working Rb expression (Scott et al., 2015). Since Rb plays such a large role in cell cycle arrest and the subsequent rate of cell proliferation, the presence of Rb may be an important factor in the proliferation rates of the two cell lines (Figure 5).

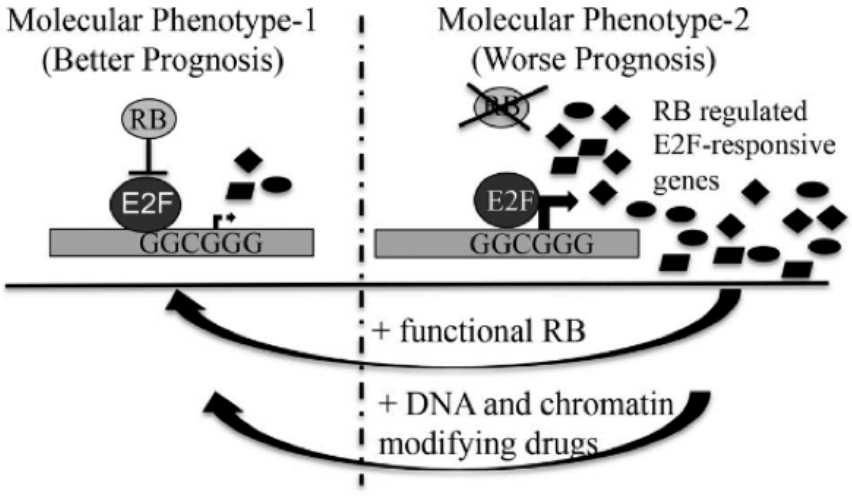

Figure 5. Osteosarcoma cell lines and Rb knock out. The OSCA 32 cells are represented by molecular phenotype 1 and the OSCA 40 cells are represented by molecular phenotype 2. The addition of ectopic Rb, or other Rb influencing drugs, can make the prognosis better. Adapted from Scott et al. (2015).

Since I3C can decrease proliferation by regulating protein expression upstream of Rb, we can observe how the lack of Rb might change the effectiveness of I3C as a chemotherapeutic drug. Because of the historically high levels of Rb mutation in studied cancer cells, it is important to understand how a drug that is dependent on the continuation of the Rb regulation, functions when the path cannot be completed. Other variant gene expression levels in the two osteosarcoma cell lines may provide even further understanding of how vast genetic differences in cancer cell lines can cause changes in their responses to known chemotherapeutics.

I3C has been proven to be an effective proliferation inhibitor and apoptosis activator in multiple cell lines. Since I3C is also known to activate the tumor suppressor protein p21 therefore inhibiting CDK6 which is involved in Rb activation, then osteosarcoma cell lines OSCA 40 and 32 and their varying ability to express Rb provide an excellent model to observe the effect a missing regulator protein has on I3C chemical treatment (Giacinti and Giordano, 2006; Mocanu, et al., 2015; Scott et al., 2015; Han, et al., 2016). The goal of this study is to investigate these osteosarcoma cell lines and develop a better understanding of the way characteristically different lineages of a single cancer type respond to treatment with I3C. Understanding these differences can help with the development of phytochemicals into chemotherapeutics, a continuing topic of discussion in current cancer research.

Materials and Methods

Cell culture and Growth

The canine Osteosarcoma (OSA) cell lines OSCA 40 and OSCA 32 were obtained from Dr. Modiano of the University of Minnesota. Cells were cultured at 37℃ in a 5% CO_2

humidified atmosphere in a sterile growth medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco), 10% fetal bovine serum (Fisher), [.02M] HEPES (Gibco), and Primocin (Invivogen). The indole-3-carbinol was purchased from Fisher, and dissolved in ethanol to form a stock solution.

Cell Proliferation Assay

100µL of cells at a concentration of $5X10^4$ cells/mL were seeded in a 96 well microplate plate and incubated in 100µL medium for two hours before I3C treatment was added. Prior to cell treatment, the I3C stock solution was diluted in 100 µL medium at twice the final concentration. Upon treatment, this dilution was added to the 100 µL of medium already in each well. The cells were treated with 0, 50, 75, 100, 300, and 500 µM I3C, and allowed to incubate in a 5% CO_2 atmosphere at 37℃ for 72 hours. The negative control, 0µM I3C, was treated with ethanol. After the cells incubated for 72 hours, 20µL of CellTiter AQ_{ueous} One Solution, a MTS Tetrazolium and PMS (phenazine methosulfanate) solution, was added to each well. The cells were incubated for another 2 hours to allow the solution to penetrate the cell membrane and enter the cell. The tetrazolium then reacts with the metabolic byproducts NADH and NADPH, found only in living tissue, and is reduced to formazan, a colored product that allows the active cell density to be read with a microplate reader at an absorbance of 492nm.

Cell Isolation

To isolate RNA for reverse transcription quantitative polymerase chain reaction (RT-qPCR), two 60mm plates were seeded to produce a surface coverage of approximately 30% of the plate and incubated for three hours. The plates were treated with either 100µM I3C or an ethanol control and incubated for 21 hours in an atmosphere of 5% CO_2 at 37˚C. The cells were removed from the plates using 3mL phosphate buffer solution (PBS) and scraping the suspended

cells into a conical tube with a rubber policeman. The cells were centrifuged at 4˚C and 1500G for 10 minutes. After transferring the pellet to a micro centrifuge tube, it was suspended in 1mL PBS and centrifuged at 4˚C and 1500G for 2 minutes. The cell pellet was stored at -80˚C.

RNA Isolation

The cells were lysed with 300µL of a 100:1 lysis buffer and 2-mercaptoethanol mixture. Then 300µL 70% ethanol was added. The samples were transferred to a spin centrifuge tube, a glass fiber filter to attract the RNA and collection tube for the waste product, and centrifuged at room temperature and 1200G for 1 minute. The supernatants were discarded and Wash Buffer I (700µL) was added to wash out any non-RNA cell components. The samples were again centrifuged at room temperature and 1200G for 1 minute. The same process was repeated twice for Wash Buffer II (500µL), in order to thoroughly wash out other types of non-RNA cell components. The samples were then centrifuged at room temperature and 1200G for 2 minutes to remove any remaining. 80µL RNase Free H_2O was added to the samples to wash the RNA off of the filter, centrifuged at room temperature and 1300G for 2 minutes. RNA concentration was determined using a NanoDrop Spectrophotometer. To ensure that only RNA had been isolated, the samples were run through gel electrophoresis using SYBR green dye. The bands were observed to determine the contents of the samples. RNA was present, but no DNA was isolated.

cDNA synthesis

Following the SuperScript® VILO™ cDNA Synthesis kit instructions, reverse transcriptase generates cDNA from the isolated RNA. A 2:1 mixture (3µL) of the 5X VILO™ Reaction Mix (primers, $MgCl_2$, dNTPs, buffer solution) and 10X SuperScript® Enzyme Mix (SuperScript® III RT, RNaseOUT™ Recombinant Ribonuclease Inhibitor, proprietary helper protein) was combined with H_2O and 200ng RNA for a total of 10µL per sample. The samples

were then placed in a thermocycler and run through the cDNA synthesis temperature cycle, which includes primer annealing at 25˚C for 10min, DNA synthesis at 45˚C for 60 min, enzyme deactivation at 85˚C for 5min, and then chilled to 4˚C. The cDNA products were stored at -80˚C before RT-qPCR.

Primer Design and RT-qPCR

The primer design was formed on an exon-exon boundary in the target gene with a melting temperature between 58˚C and 60˚C, determined by the formula Tm=60.3+(0.41*%GC) –500/n, where n is the nucleotide length of the primer. The products of the PCR reactions using the primers were between 140 and 200 base pairs. The primers used in this study included one set for the CDKN1A gene (5'-TGAGGCATCCCCGGGTGT-3'/5'-TGTAGAAGGGGCCTGGGATG-3') and two sets for the Rb1 gene (5'-CCGAGAAGGACCAGCTGATC-3'/ 5'-GTCTGGAAGGCTGAGGTTGC-3' and 5'-GGAAAAAGGAGCTATGGGGAATCTG-3'/ 5'-GAGTGCACACAATACATCGTACTTC-3'). The control primer set was from the HPRT1 gene (5'- GATGAAGGAAATGGGAGGCC-3'/ 5'- GTCCCCTGTTGACTGGTCAT-3'). Forward and reverse primers were diluted in RNase Free H_2O to form a 100mM solution and added together (10µL each) to RNase Free H_2O (80µL) to form the PCR primer solution.

70.2µL of a Primer/SYBR Green mixture (100µL 2XSYBR Green, 90µL RNase Free H_2O, 5µL target primer solution) was added to 1.8µL of the target sample cDNA. The cDNA was then amplified, in triplicate for each primer set and treatment sample combination, using polymerase chain reactions (PCR) in a cycle of high temperature (95℃) denature the DNA into single strands, and a lower temperature (70-72℃) to optimize DNA polymerase and primer extension.

Statistical Analysis

Statistical analysis for the cell proliferation assays was done using a Random Block ANOVA test and a Tukey HSD test, differences were considered significant at $p<0.05$. For PCR, statistical analysis was done using a Two Tail Student t-Test

Results

I3C Inhibits Cell Proliferation

Two osteosarcoma cell lines with unique molecular profiles, OSCA 32 and OSCA 40, were treated with the same I3C concentrations to observe how these molecular divergences affect the efficacy of chemical treatment. Both cell lines were treated with varying concentrations of indole-3-carbinol or ethanol, proliferation was measured after 72 hours of treatment. For each cell line, I3C caused a significant decrease in proliferation (Figure 6). However, there was a difference in how the concentrations of I3C decreased proliferation. For OSCA 40 a correlation appears between the percent of effect on proliferation and the concentration of I3C, as the treatment levels were significantly different from each other. This appears to be less applicable in the case of OSCA 32, where there was not a significant effect until the concentration was at least 100 μM, while for OSCA 40, treatment with 50 μM I3C caused an initial significant decrease.

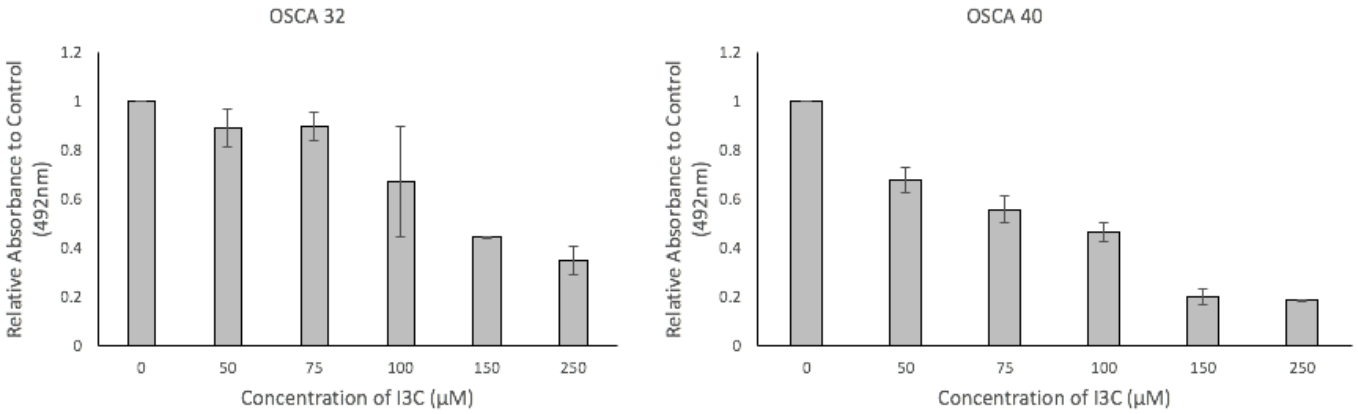

Figure 6. Proliferation of OSCA 40 and OSCA 32 decreased after I3C treatment. Both OSCA 32 (A) and OSCA 40 (B) cells were treated with several concentrations of indole-3-carbinol (0-250µM). After 72 hours of treatment, proliferation was measured using the CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay kit. Proliferation values are measured in a direct relationship by absorbance (492nm). A Random Block ANOVA test supported significant differences in cell proliferation within the treatments ($p<0.05$). The letters above each data point represent the varying significant differences using a Least Square Means Tukey HSD test. A significant difference in proliferation is represented between each letter.

RT-qPCR Reveals an increase in p21 expression

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR) was performed on OSCA 32 and OSCA 40 cell samples treated with both 100µM I3C and ethanol as a control. RT-qPCR investigated the levels of RB1 and CDKN1A mRNA in each sample and how I3C altered these levels. Two RB1 primers, labeled Rb1 and Rb2 were used. For both cell lines, I3C caused no apparent increase in RB1 or CDKN1A mRNA levels was observed for either primer set (Figure 7).

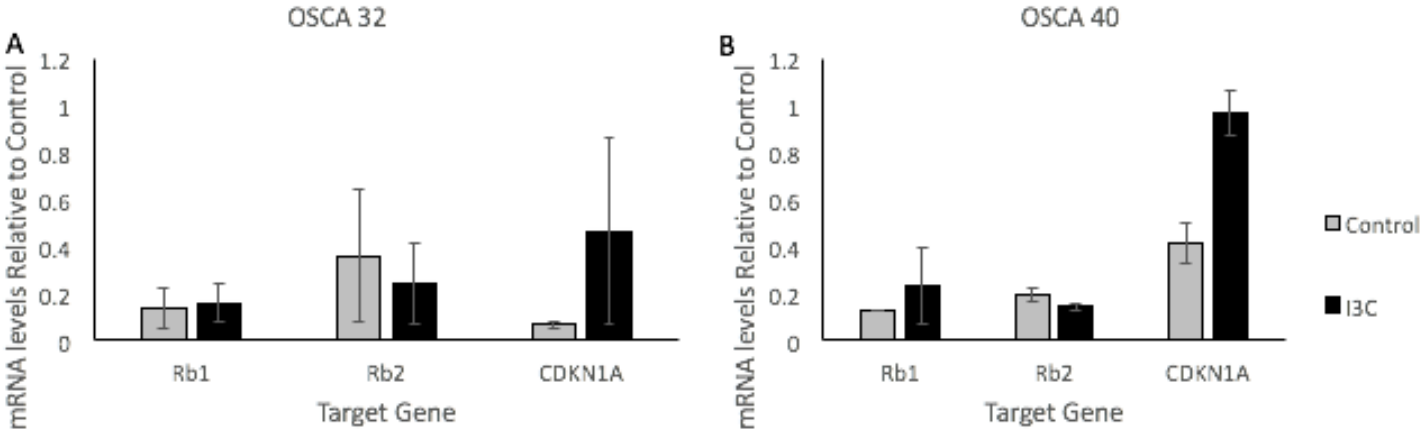

Figure 7. I3C increased CDKN1A expression in OSCA 32 and OSCA 40. Both OSCA 32 (A) and OSCA 40 (B) cell lines were treated with 100μM I3C or an ethanol control. After 21 hours, the cells were lysed and the RNA extracted. cDNA was synthesized from the extracted RNA, and target gene primers amplified the target mRNA. Relative mRNA expression is calculated by average $2^{-\Delta Ct}$ between the target mean Ct and the control primer (HPRT1) mean Ct. A Two Tail Student t-Test was used to analyze significant difference between mRNA levels in treated and control cells. No significant differences were found.

Discussion

Biologically active chemicals found in plants have been observed to decrease proliferation of cancer cells due their ability to affect a variety of pathways within tumor cells. However, very few of these chemicals have been studied intensively (Hosseini and Ghorbani, 2015). Indole-3-carbinol (I3C) has most widely been studied for its effects on the G_1/S cell cycle arrest checkpoint, specifically on the upregulation of the p21 and p53 tumor suppressor proteins (Chinni et al., 2001; Choi et al., 2010). However, previous research has not included any osteosarcoma cell lines. Observing multiple osteosarcoma cell lines with unique molecular profile can provide interesting insight into the influence that molecular mutations have on the efficacy of chemical treatments. The OSCA 32 cell line has normal retinoblastoma protein (Rb) expression levels and therefore has a potentially differently functioning G_1/S checkpoint.

However, mutations in OSCA 40 cause inactivation of the Rb gene and a non-functional G_1/S checkpoint due to a lack of Rb protein (Scott et al., 2015). This study was set up to observe the impact I3C has on cells with varying functionality in the suspected target pathway of I3C.

Based on previous research implying the ability of I3C to upregulate G_1/S cell cycle arrest, and the disrupted G_1/S pathway in OSCA 40 cells due to inactive Rb expression, it was hypothesized that I3C would have a diminished effect on OSCA 40 proliferation compared to OSCA 32 proliferation. Unexpectedly, in this study, it was observed that both OSCA 40 and 32 were affected by I3C treatment. In fact, of the two cell lines, I3C was most effective against proliferation of the OSCA 40 cell line (Figure 6). A closer look into the mRNA levels of the RB1 and CDKN1A genes provides insight into the expression of the protein products of these genes, Rb and p21, and their role in proliferation. Interestingly, the change in mRNA levels of p21 and Rb after treatment with I3C was similar between the two cell lines. In both OSCA 32 and OSCA 40 there were no effects on Rb or CDKN1A levels. Since p21 mRNA was not regulated upon treatment, it can then be surmised that the anti-proliferative effects I3C has on OSCA 40 and OSCA 32 are not due to G_1/S checkpoint regulation. This data then suggests that I3C treatment modifies other anti-proliferative pathways in osteosarcoma cells.

Other studies have observed that I3C may be involved in the induction of cell cycle arrest at the G_2/M cell cycle checkpoint. In a study using human colon cancer tissue (SW480), it was shown that I3C significantly increased the accumulation of cells in the G_2/M phase (Hudson et al., 2003). Looking specifically at OSCA 32 and OSCA 40, it has been observed that due to mutations in the Rb functionality in OSCA 40 cells, there is an upregulation in E2F-regulated genes. Some of these upregulated genes are involved in the G_2/M checkpoint (Scott et al., 2015). Therefore, decrease in cell proliferation due to I3C may have been higher in OSCA 40 due to the

increased G_2/M regulatory genes affected by I3C. This information provides support for future studies on the varying effects of I3C on G_2/M regulation in OSCA 32 and OSCA 40 cell lines. These studies could also narrow down the function of I3C found within the cell, and how the molecular profiles of these cells change the efficacy of treatment.

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