The dose and time dependent regulation of IGF-1R in MCF-7 breast cancer cells by indole-3-carbinol

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Abstract   Breast cancer is the most common cancer among women. The development and progression of cancer cells can often be attributed to problems with cell signaling pathways, including pathways of growth, differentiation, proliferation, and apoptosis. The IGF (Insulin-like Growth Factor) pathway is one such pathway where signaling can contribute to breast cancer cell survival. The side effects and drug resistance associated with current treatment options have led to research regarding the potential of phytochemicals as a treatment option for cancer. Indole-3-Carbinol (I-3-C) is a promising phytochemical found in cruciferous vegetables. Research has shown consistent results regarding a positive correlation between cancer prevention and the consumption of such phytochemicals. Reverse-Transcription (RT) PCR and Western Blot analyses were used to examine the potential effects of I-3-C on the IGF pathway. A decrease is observed in the transcript levels of IGF-1R with increasing doses of I-3-C. This indicates that I-3-C can be used to decrease, or eliminate, the activity of the MAP kinase pathway (of the IGF pathway), hence decreasing proliferation of tumorigenic MCF-7 cells.

Introduction

Cancer is the second leading cause of death in the United States (ACS 2006)[1] and breast cancer is the second leading cause of death among women in North America (Smigal et al. 2006). Compared to normal body cells that undergo a cycle of growth, division, and in time, death, cancer cells continue their growth and division, and form new abnormal cells (ACS 2006). These cells can metastasize, which is when they travel to other parts of the body, grow, and replace normal tissue (ACS 2006). Cancer arises due to heritable damage, or mutations, to DNA. While the body is able to repair most damage to DNA, mutations that persist are deleterious. Treatment of cancer involves surgery and radiation where the goal is to treat a tumor without affecting other parts of the body (ACS 2006). Treatment also includes chemotherapy, immunotherapy, and hormone therapy (ACS 2006). According to Hakimuddin et al. (2006), systemic treatments[2] for cancer can have severe side effects, and sometimes become ineffective when cancer cells become resistant to drugs. The side effects and drug resistance have led to an interest in alternative approaches to cancer treatment.

Cancer can be a genetic and a lifestyle disease. Therefore, prevention is linked to changes in lifestyle, such as dietary practices. Recommendations include a reduced intake of salt, fats, and alcohol (Tominaga 1990; Hakimuddin et al. 2006), with increased uptake of fruits and vegetables (Hakimuddin et al. 2006). These approaches include the incorporation of natural components such as phytochemicals[3] from the diet (Hakimuddin et al.; Moreno et al. 2006). Indole-3-carbinol (I-3-C) is one of the many phytochemicals (Moreno et al. 2006) being considered as an alternative cancer treatment approach. It is found in cruciferous vegetables (family Brassicaceae) such as broccoli, cauliflower, cabbage, and brussel sprouts (Cover et al. 1998, 1999; Moreno et al. 2006; Sundar et al. 2006). Cruciferous vegetables have been linked to reduced risk of cancer (Moreno et al. 2006; Wargovich 1999), and are quite well recognized candidates for health promoting compounds. Studies regarding the active components in such vegetables, such as I-3-C, consistently show a positive correlation between cancer prevention and consumption of these active constituents (Moreno et al. 2006).

Despite the extensive research surrounding indole-3-carbinol, its effects on many cell signaling pathways are still unknown. Signal transduction pathways regulate processes such as cell growth, proliferation, and cell death (Hakimuddin et al. 2006). Cancer development and progression can

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[1] The leading cause of death, for men and women, in the United States is heart disease (ACS 2006).
[2] Systemic treatment refers to chemotherapy, immunotherapy, and hormone therapy (ACS 2006).
[3] Phytochemicals are substances, or compounds, of plant origin; plants use them as part of their own natural defense against disease and age (Wargovich 1999).

often be associated with problems along the signaling pathway; for example, breast cancer has been linked to uncontrolled activation of the mitogen-activated protein kinase[4] (MAP kinase) and the phosphatidylinositol-3 (PI-3) kinase pathways (Hakimuddin et al. 2006; Moschos and Mantzoros 2002; Yu and Rohan 2000). Based on the positive correlation for cancer prevention, I-3-C is being considered as a contender to target growth factors, such as insulin-like growth factors (IGFs), which are involved in regulating cell growth, differentiation, apoptosis and transformation. This is an important consideration as the imbalance of the normal cell cycle processes can lead to malignant transformation of cells (Moschos and Mantzoros 2002; Yu and Rohan 2000).
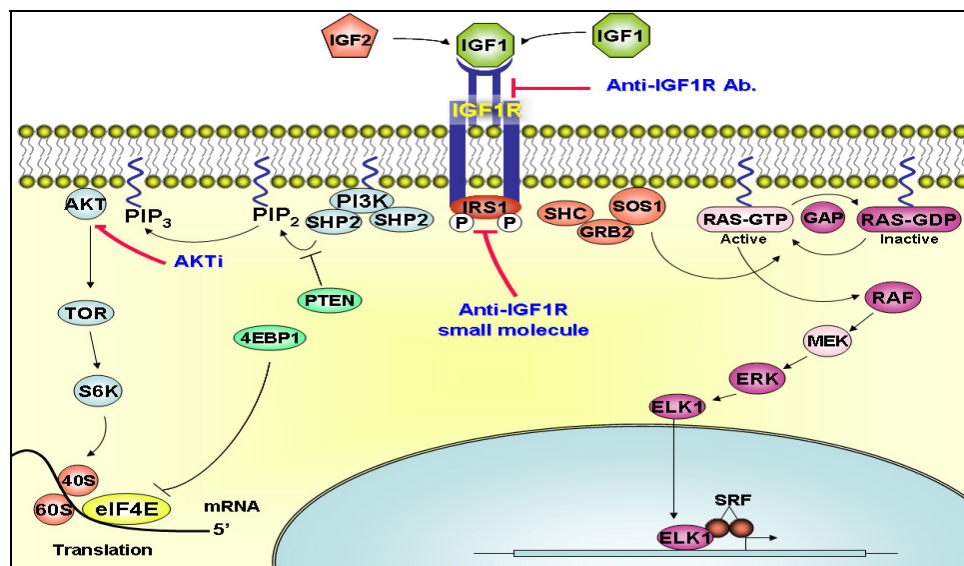
(Center for Cancer Research 2007)
Figure 1: Cartoon of the IGF pathway, showing the MAP kinase (right) and PI-3 kinase (left) pathways. When the IGF-1 molecule binds to its receptor, IGF-1R, both transcription (MAP kinase) and translation (PI-3) are activated, leading to survival of tumorigenic cells.

The IGF family has two ligands, IGF-I and IGF-II, and their receptors, IGF-IR and IGF-IIR (Moschos and Mantzoros 2002; Yu and Rohan 2000). When IGF binds its receptor, the receptor autophosphorylates, activating the IGF pathway (Moschos and Mantzoros 2002), including the MAP kinase pathway, and the phosphatidylinositol-3 (PI-3) pathway (Figure 1). The MAP kinase pathway is associated with growth and proliferation, while the PI-3 kinase pathway prevents differentiation and apoptosis, leading to the survival of tumorigenic cells (Moschos and Mantzoros 2002). According to Moschos and Mantzoros (2002), IGF-I enhances division of breast cancer cells,

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[4] Kinases are enzymes that add phosphate groups (PO_4) to molecules, in most cases, activating them.

and IGF-I receptors can be detected more frequently in breast cancer cells than in normal cells. Furthermore, if the IGF-I receptor were removed from the signal transduction pathway, preventing interactions with IGFs, the mitogenic activity of these growth factors could be eliminated (LeRoith et al. 1995). Yet to be examined, however, are the effects of the down-regulation of IGF-1R (when complete elimination is not possible) on the survival and proliferation of tumorigenic cells.

This study measured the ability of indole-3-carbinol to interrupt the proliferation of tumorigenic breast cells, where dosage effects of I-3-C on IGF-1R were monitored at different time points, representing different phases of the cell cycle. The prediction is that indole-3-carbinol down-regulates the activity of IGF-1R (insulin-like growth factor-1- receptor) in a dose-dependent manner, with response to time.

Methods

The effects of I-3-C were tested on MCF-7 cells. These are estrogen responsive breast cancer cells, and I-3-C transcriptionally down-regulates estrogen receptor-alpha[5] (ER-α) expression in these cells (Sundar et al. 2006).

Tissue Culture and Treatment MCF-7 cells were seeded into 10cm plates, with 6mL of growth media per plate. The growth media consisted of 500mL DMEM (Dulbecco's Modified Eagle's Medium), with 10% FBS (Fetal Bovine Serum), 1% L-glutamine, 0.25% PennStrep (Penicillin Streptomycin), and 0.1% insulin. The cells were incubated at 37°C, and the media was changed every two days.

I-3-C was dissolved in 99.9% dimethyl sulfoxide (DMSO) for administration, therefore DMSO served as the vehicle control for the experiment (Cover et al. 1998 and 1999). When cells reached 50-60% confluency, 1μL/1mL (Cover et al. 1998 and 1999) of I-3-C, in 50mM, 100mM, 200mM, and 300mM concentrations, was applied to the cells in growth media. The cells were incubated, with the drug in the media, for the intervals of 24h, 48h, and 72h periods. They were then harvested, and RNA and protein from each sample were isolated using the provided protocols (Crystal Marconett and Shyam Sundar, University of California, Berkeley, personal communication).

Reverse Transcription-Polymerase Chain Reaction RT-PCR was used as to examine the transcriptional aspect of the pathway (Figure 1). Ribonucleic acid, RNA, was isolated (Appendix 1), converted to DNA via reverse transcription, and then amplified using PCR (Appendix 1). [PCR does not increase the amount of RNA or DNA in the sample; it merely amplifies the number of copies of

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[5] Estrogen Receptor has two isoforms, ER-α and ER-β.

the existing DNA so that gel electrophoresis can be done.] The parameters tested in PCR were GAPDH, a loading control, ER-α, the positive control, and IGF-1R. The PCR products were then run on a 1% agarose gel (1g of agarose per 100mL of buffer). GAPDH serves as the loading control, meaning it indicates the quality of RNA quantification, hence the quality of loading. Uniform loading is indicated by the evenness of GAPDH bands as I-3-C has no effect on GAPDH. We compare the loading of the GAPDH samples to the ER-α and IGF-1R samples to determine whether differences in band intensities are due to the effects of I-3-C, or due to unequal loading. Down-regulation of IGF-1R was based on comparisons between the ER-α band patterns, and the loading control. Down-regulation was indicated by the bands getting fainter, and thinner compared to the DMSO and the loading control bands.

Western Blot Analysis Western blots measure protein levels, hence are a way for examining translational aspects (Figure 1) of indole-3-carbinol's effects on the Insulin-like Growth Factor pathway. Protein was isolated from the samples (Appendix 2), and quantified using the Bradford method of standardization (Crystal Marconett and Shyam Sundar, University of California, Berkeley, personal communication). Protein is quantified and standardized to ensure uniform loading of the samples onto the gel for electrophoresis. Actin and ER-α were used as controls; ER-α is used as a positive control and actin is used as a loading control. Actin is a motor protein, hence is expressed uniformly in all cells, meaning there are no differences in actin levels amongst the different samples. Down-regulation of protein levels was determined in a similar manner as down-regulation of RNA levels, where band intensities were judged on their prominence, compared to the loading control, and amongst the concentration of the drug in each sample.

Results

Indole-3-Carbinol causes a decrease in IGF-1 receptor's transcript levels, in a dose and time-dependent manner (Figure 2). The band for DMSO is the brightest and thickest, and then the bands become increasingly faint and thin as the concentration of I-3-C increases; the most noticeable difference is between DMSO and 200mM I-3-C, in the 48h and 72h trials (Figure 2). Down-regulation of IGF-1 receptor correlates to the positive control, ER-α, where a pattern is observed quite clearly with increasing doses of I-3-C, in response to time.

The effects of indole-3-carbinol on protein expression levels of IGF-1 receptor are unclear (Figure 3). Actin levels indicate equal/uniform loading. Down-regulation of ER-α is seen in a dose-dependent manner, where the band for DMSO is the most prominent, and then the bands become

fainter with increasing doses of I-3-C (Figure 3). This is observed for both 24h and 48h trials. However, not much can be determined about protein expression levels of IGF-1 receptor, for either timepoint. For the 24h interval, the bands for DMSO, 100mM, 200mM, and 300mM look about the same; for the 48h interval, the DMSO band looks fainter than all the I-3-C bands (Figure 3).
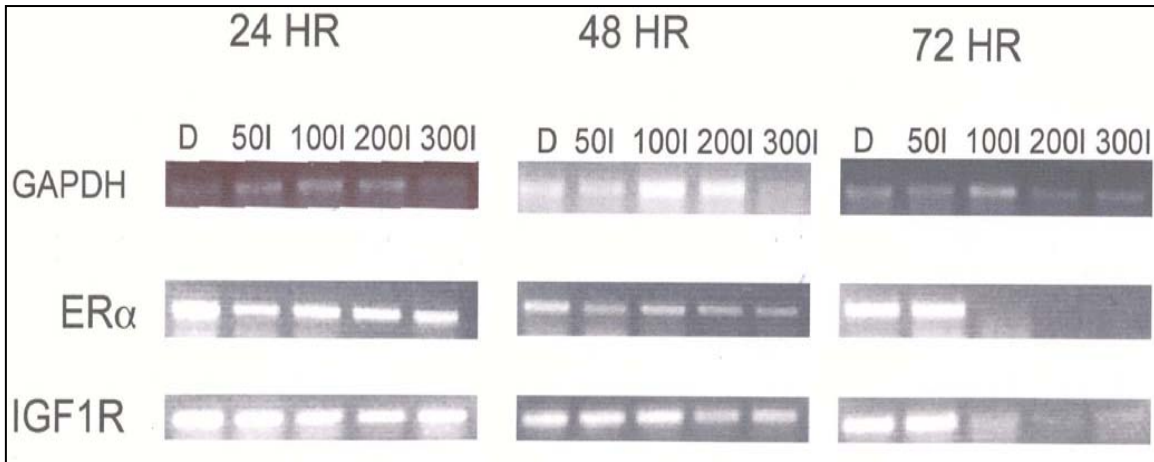

Figure 2: RT-PCR showing transcript levels of IGF-1R in MCF-7 cells for 24, 48, and 72h intervals for four doses of I-3-C (50, 100, 200, 300 mM), with negative control DMSO. D=DMSO; I=I-3-C. GAPDH is loading control; ER-α is positive control.
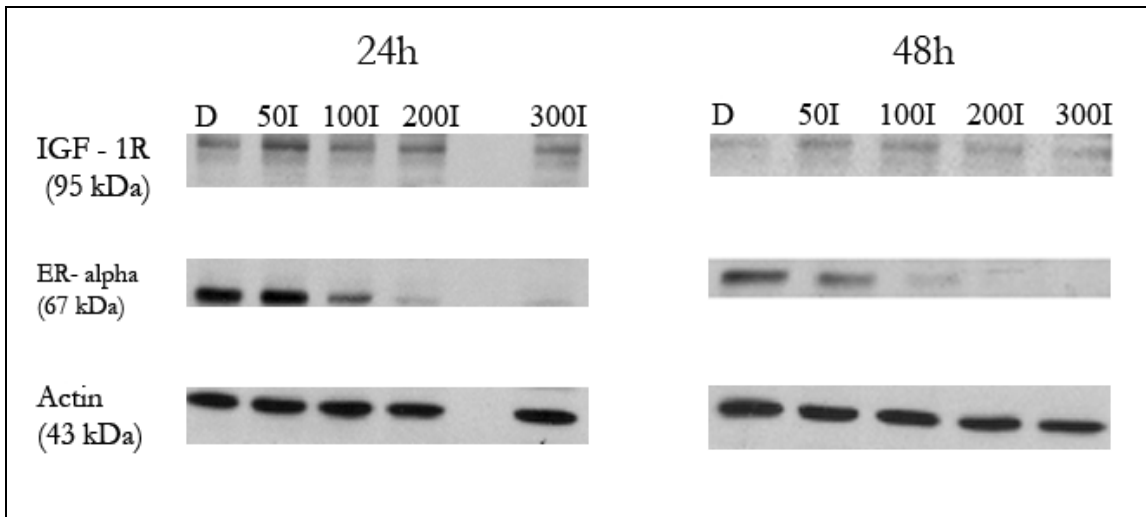

Figure 3: Western blots showing protein expression levels of IGF-1R in MCF-7 cells for 24h and 48h intervals, with four doses of I-3-C (50mM, 100mM, 200mM, 300mM). D=DMSO, negative control; I=I-3-C. Actin is loading control, ER-α is positive control. Note: Western blots were not carried out for the 72h interval.

Discussion

This study investigated the dose- and time-dependent effects of indole-3-carbinol on the insulin-like growth factor (IGF) pathway. The drug was used in an attempt to interrupt the survival of the

tumorigenic cells. It was found that I-3-C down-regulated transcript levels of IGF-1 receptor with the strongest decrease at the 200mM dose. This is supported by the qualitative observation where cells stopped proliferating on tissue culture plates, with increasing doses of I-3-C.

Similar results were expected for protein levels, given the link between indole-3-carbinol, ER-α, and MCF-7 cells (Sundar et al. 2006). Protein levels of the IGF-1 receptor were expected to decrease, as was observed with the positive control, but this was not the case. IGF-1R protein levels looked almost uniform for the intervals tested (24h and 48h), for different doses as well as the time intervals tested. Due to time and resource constraints, and the inconclusive results of the 24h and 48h trials, Western blot analysis was not carried out for the 72h interval.

Problems with Western blots are associated to the sensitivity, or specificity, of the antibodies used. In several trials of Western blots, similar issues were encountered, where sometimes bands did not appear at all. This is attributed to errors in technique, where the blots were not sufficiently incubated in their antibodies.

Based on the transcriptional down-regulation of IGF-1R by I-3-C, further studies can examine the effects of the drug on aspects of the pathway that are downstream of IGF-1R (Figure 1). The findings of this study can also be used to examine other growth factor pathways, such as the Epidermal Growth Factor (EGF) (Nicolini et al. 2006) and the Vascular Endothelial Growth Factor (VEGF) (Nicolini et al. 2006, and Schneider and Sledge 2007). In addition, it is possible to test the effects of I-3-C in combination with other phytochemicals, such as Artemisinin and DIM (3'-3'-dindolylmethane, a dimer of I-3-C), on such growth factor pathways.

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Appendix 1

RNA Isolation

To collect RNA, growth media was aspirated and 1mL Trizol reagent (Sigma Aldrich) was added to the cells. The cells were then scraped and transferred to 1.5mL RNase free eppendorf tubes. They were allowed to digest in Trizol reagent for 5 minutes at room temperature. To this, 200μL chloroform was added and the samples were shaken vigorously. They remained at room temperature for 5 minutes, and then were centrifuged for 30 minutes at 14, 000 rpm at 4°C. The clear supernatant containing the RNA was transferred to new, RNase free, eppendorf tubes. To this, 500μL isopropyl alcohol (100%) was added, and the samples were placed at -20°C for at least 30 minutes [or stored at -80°C overnight]. They were then centrifuged for 20 minutes at 14,000 rpm at 4°C. After aspirating the isopropyl alcohol, the pellets were resuspended in 1mL 75% ethyl alcohol, centrifuged for 20 minutes at 14,000 rpm at 4°C, and then air-dried until the pellets turned clear. These pellets were resuspended in 100μL RNase free, deionized water. The samples were stored at -80°C until Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Reverse Transcription-Polymerase Chain Reaction

For reverse transcription, 25μL of the isolated RNA was transferred to a PCR tube, to which reverse transcription buffer, 10μM deoxynucleotides (dNTPs), random hexamers, reverse transcriptase (enzyme), and RNase inhibitor were added. The samples were put on a 70°C heat block for 5 minutes, and then placed in a thermocycler where they were at 42°C for 1.5h. The samples were stored at 4°C until PCR. The PCR was programmed such that the samples were at 94°C for one minute, 56°C for thirty seconds, and at 72°C for thirty seconds. The cycle was repeated 37 times.

Appendix 2

Protein Isolation

Cell pellets for protein isolation are obtained much the same way as they are for RNA isolation. Growth media was aspirated, cells were scraped in 1mL PBS and transferred to 1.5mL eppendorf tubes. The cells were centrifuged at 12,000 rpm in PBS for 5 minutes at room temperature. The PBS was aspirated and each pellet was resuspended in 200μL RIPA with protease inhibitors. They were resuspended using a 21-G needle in a 1mL syringe (to lyse cells in order to extract protein), and then centrifuged at room temperature for 10 minutes at 12,000 rpm. After centrifugation, the supernatant was transferred to new 1.5mL eppendorf tubes and stored at -80°C until gel electrophoresis for Western Blot analysis.

Western Blot Analysis

After quantification, protein from each sample was alliquoted with RIPA [including protease inhibitors], and gel loading buffer. These were boiled on a 100°C heat block for 5 minutes to unfold the proteins in the sample before loading onto 6% acrylamide gels. After separation by electrophoresis, the gels were transferred onto nitrocellulose membranes, and then cut into sections, based on size, to probe for IGF-1R, ER-α (positive control), and actin (loading control), using primary and secondary antibodies. [Primary antibody has binding specificity to the protein of interest (IGF-1R, ER-α, and Actin), and the secondary antibody has specificity for the primary antibody.