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Regulation of Anti-Proliferation in Melanoma and Breast Cancer Cells by Small Molecule Phytochemicals and Their Derivatives

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Regulation of Anti-Proliferation in Melanoma and Breast Cancer Cells by Small Molecule Phytochemicals and Their Derivatives

By

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University of California, Berkeley

Committee in charge:

Professor Gary L. Firestone, Chair
Professor Dale Leitman
Professor Terry Machen

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Abstract

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Professor Gary L. Firestone, Chair

Melanoma and breast cancer represent a significant portion of overall cancer burden, and many strategies have been developed to control their growth. Many of these current options have serious adverse effects and sometimes lead to therapy resistance which can complicate future treatment. Because of this, it is critical to investigate new classes and combinations of therapeutics that can overcome these drawbacks and retain effectiveness in multiple phenotypes. This thesis details the mechanism in which the combination of Indol-3-Carbinol (I3C) and Acetylsalicylic Acid (aspirin) act synergistically to slow the growth of human melanoma cells. In addition, this thesis demonstrates that the phytochemical Artemisinin and its derivative, Artesunate are able to inhibit the growth of luminal A estrogen receptor positive breast cancer cells. Finally, we utilize I3C derivatives to analyze the growth effects of different chemical functional groups in both melanoma and breast cancer cells. The combination of I3C and aspirin was able to synergistically control melanoma cell proliferation through an induction of G_1 cell cycle arrest. Cell cycle arrest was due likely to a similar downregulation of Cyclin Dependent Kinase 2 (CDK2) levels which in turn is under the transcriptional control of the Microphthalmia-Associated Transcription Factor M (MITF-M). The combination of I3C and aspirin was able to significantly downregulate MITF-M protein and transcript expression. Luciferase analysis of the proximal region of the MITF-M promoter revealed that both compounds regulated promoter activation synergistically. The reduction in activation was mediated by two distinct pathways that met on the MITF-M promoter, I3C inhibited the activation of BRAF which caused a decrease in binding to the promoter of the transcriptional activator BRN-2 while aspirin downregulated β-catenin/LEF1 binding to the promoter, again decreasing activity. Site directed mutagenesis of these sites proved that they were sufficient for the compounds' regulation of MITF-M transcriptional activity. These results uncover one mechanism in which aspirin controls melanoma growth, something that has not been explored, and the combination of aspirin and I3C could be utilized as a potential anti-cancer treatment in human melanoma. We also demonstrate that Artemisinin, a phytochemical derived from *Artemisia annua* and its derivative Artesunate are able to regulate the growth of human breast cancer cells through regulation of Cyclin A2, and cyclin dependent kinases 1 and 2 (CDK) critical regulators of the G_1/M phase transition as well as G_2 phase progression. Artesunate was able to achieve significant downregulation and induce growth effects at much smaller doses than its parent molecule indicating increased potency. Chromatin-immunoprecipitation indicated that Artemisinin was

able to alter Sp1 binding to the Cyclin A promoter, potentially explaining its ability to regulate transcript levels. In the last chapter, we explore the effects on melanoma and breast cancer cell growth caused by 1-Benzyl-I3C derivatives. We find that small changes in structure alter growth inhibition by the compounds likely through changes in their ability to interact and alter function of target enzymes Human Neutrophil Elastase, and NEDD4-1. No compound was stronger than the parent molecule, 1-Benzyl-I3C, but all were able to induce significant changes in growth at concentrations lower than I3C. While this study is preliminary it provides information as to what potential derivatives might slow transformed cell growth in the future.

Acknowledgments

First and foremost, I would like to thank my mentor, Professor Gary Firestone. You allowed me to join the lab after working as an undergraduate student, and guided me throughout my studies while at the same time giving me the space necessary to develop my independent drive and motivation. It has been a wonderful experience having a PI that I can come to both for suggestions on my project as well as to discuss the latest NFL news. I have learned so much from you about thinking in a critical and unbiased way, and will retain these lessons for the rest of my professional career. Thank you for everything, Gary.

Kelly – It is really hard to believe that after meeting you in Gary's lab at the end of our undergraduate studies, we would find ourselves together now. Your endless support and encouragement through the times in which I doubted myself and what I was doing is what helped me push through to this point. Your combination of poise, humor, kindness, and intelligence has never ceased to amaze me, and never will. Thank you so much for putting up with me and helping me carry the load, I can't wait to see what we accomplish together.

Crystal - You took me under your wing many years ago and showed me the ropes. You were one of the best teachers I have ever had, with a great combination of knowledge, patience, and skill, you put up with the mistakes I made and helped me develop into a functional graduate student. I learned so much from you and always admired your passion for science and your quiet determination, and am so happy to see you succeed in your career.

Ankur – Like Crystal, you welcomed me into the lab not only as a friend, but as a brother. We have shared so much together, whether it was learning from you the wrong way to attach the electrodes for a DNA gel, to road-tripping to Vancouver, to going to Triple Rock with Travis. You are one of the very few people who I know will give it to me straight, and you will always be one of my best friends.

Tony – Your ability to produce great data without ever seeming to be stressed out has always been one thing about you that has amazed me. Your light-hearted, but passionate nature is a refreshing thing to see in a scientist and a friend. I am glad you ended up in Gary's lab and that we have been able to be close all of these years, and hope that since you have left the lab your mental math skills have improved.

Jeanne – Your smile and your effervescent energy have been the most consistent things in the lab since you arrived from the East Coast. Thank you for being my psychologist, colleague, and friend. Your confidence to drive biochemical projects in a lab geared towards cell biology has always amazed me. Your sarcasm and semi-regular pranks at my expense have always kept me on my toes, and made the last few years that much more enjoyable.

To my friends and colleagues in the Firestone lab both past and present; thank you all very much for creating an environment full of discovery and friendship. Working with you over the years has helped me develop new perspectives on scientific problems both big and small. Thank you all for sharing stories, samples and reagents, I wish you all the best of luck in your careers.

This work is dedicated to my parents

Michael and Sandra Poindexter

The love and support that you have given me throughout my life has allowed me to pursue my passions. I will never forget the sacrifices you both made to make sure that I succeed. Thank you for always believing in me.

Table of Contents

Chapter III

Chapter IV

List of Figures

General Introduction Figures

Chapter I Figures

Chapter II Figures

Chapter III Figures

General Introduction

Acetylsalicylic Acid

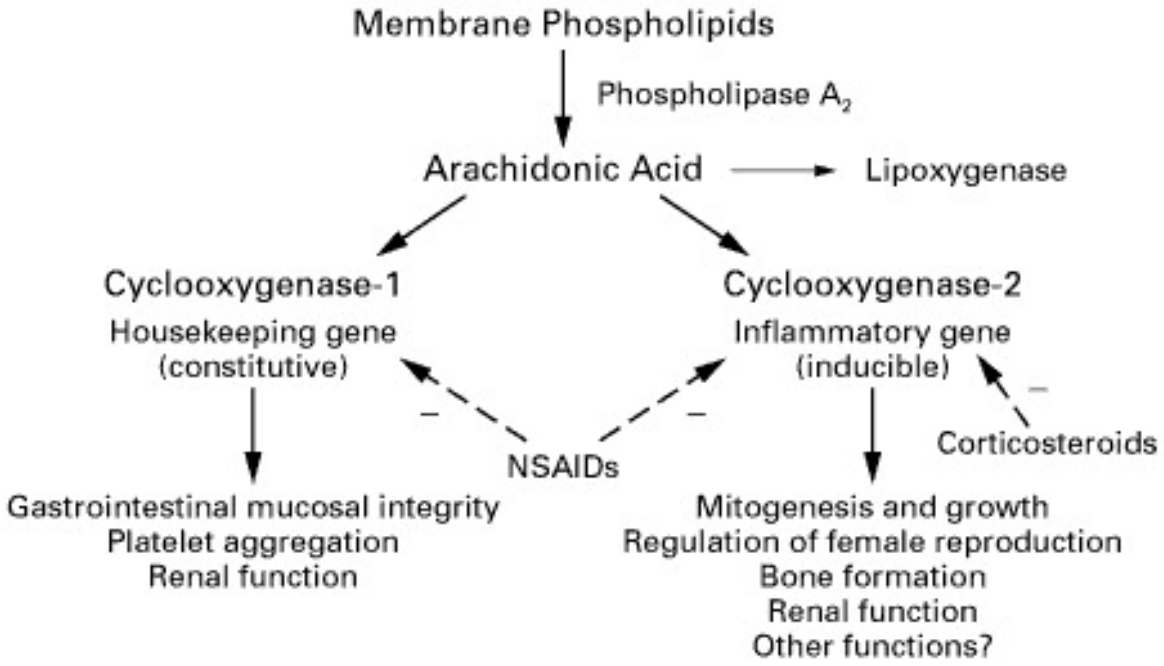
Acetylsalicylic Acid (aspirin) is the first member of the group of drugs known as non-steroidal anti-inflammatory drugs (NSAIDs), and is the acetylated derivative of the phytochemical salicylic acid, a compound originally found in willow bark (1). Aspirin has been used for over a century has many beneficial properties acting as an analgesic, anti-pyretic, anti-thrombotic and anti-inflammatory. Aspirin and other NSAIDs have been shown to work through a similar mechanism to achieve these effects, primarily through the irreversible inhibition of the cyclooxygenase 1 and 2 enzymes (COX1/2). Both enzymes act to convert the prostaglandin precursor arachadonic acid (AA) to prostaglandins. COX-1 acts as a housekeeping gene and its products protect the gastric mucosa, and activates platelets (2). By inhibiting activation and aggregation of platelets, aspirin inhibition of COX-1 explains both cardioprotective effects such as reducing chances of heart attack and stroke when taken in low doses (typically 81mg/day) for an extended period of time (3). High doses of aspirin are known to have several worrisome side effects, so reduction in arterial thrombosis comes with an increase in bleeding in the upper gastrointestinal tract (4). COX-2 is an enzyme induced by inflammation and tumorigenesis, and generates pro-inflammatory mediators that are responsible for fever, inflammation and swelling (5) (Figure 1).

More recently, many drugs have been developed to be specific COX-2 inhibitors, allowing for the elimination of the inflammatory response, while at the same minimizing gastrointestinal side effects. However, clinical trials utilizing these drugs has found that they increase the risk of certain cardiovascular events, specifically myocardial infarct and ischemic stroke. The inhibition of COX-2 also prevents the synthesis of prostaglandins responsible for vasodilation and inhibits platelet aggregation which explains such increase in risk (6). There are clear risks that need to be considered when considering aspirin as a therapy, however more often than not the risk is worth the reward.

Figure 1:

From (4): **The cyclooxegenase enzymes utilize arachidonic acid for different functions.**

COX-1 is expressed at consistent levels and helps maintain organ homeostasis by controlling mucous and acid in the stomach, body temperature, renal function, and platelet aggregation. COX-2 induces pro-inflammatory agents, vasodilatation, and inhibits platelet aggregation. NSAIDs inhibit both enzymes but at different concentrations.


Membrane Phospholipids
Phospholipase A2
Arachidonic Acid
Lipoxygenase
Cyclooxygenase-1
Housekeeping gene
(constitutive)
Cyclooxygenase-2
Inflammatory gene
(inducible)
NSAIDs
Corticosteroids
Gastrointestinal mucosal integrity
Platelet aggregation
Renal function
Mitogenesis and growth
Regulation of female reproduction
Bone formation
Renal function
Other functions?

Aspirin and Cancer

Since the early 1990's aspirin has been studied as a possible chemopreventative agent in multiple cancer subtypes in *in vitro and in vivo* studies as well as clinical studies. Meta-analysis studies of patients have demonstrated that long term, daily intake of low-dose aspirin (75mg) lowers overall long-term cancer incidence and mortality in men and women by 20% or more (7,8). While these chemopreventive effects have been demonstrated in multiple cancer types such as gastrointestinal cancers, oesophagal, pancreatic, brain, and lung cancer, these benefits have been studied in greater detail in colorectal cancers.

In one study, 5 year treatment with 300mg aspirin was able to reduce colorectal cancer incidence, but only after a latency period of 10 years (9) Other studies found similar results using data from the Nurse's Health Study (NHS), though a latency period was not discussed (10). Beyond colorectal cancer, a recent meta-analysis indicates that aspirin use might reduce pancreatic cancer rates as well as pancreatic cancer stem cell properties (11,12). Given the significance of these results plus ease and low-cost of treatment with daily aspirin, a conversation amongst clinicians to think about using aspirin as a prophylactic measure against colorectal cancers even considering the modest increase in side effects centered around gastrointestinal bleeding. While the clinical data in support of aspirin's anti-colorectal cancer ability has been mounting for some time, the mechanism in which it acts is still being studied using colon cancer cell or tumor models. These *in vitro* systems have demonstrated that aspirin can inhibit growth and induce apoptosis through a variety of possible mechanisms such as altering the cellular distribution of the NFκB complex promoting apoptosis through the downregulation pro-survival markers such as B-Cell Lymphoma 2 (Bcl-2) (13), preventing angiogenesis, and interfering with the Wnt/β-Catenin pathway (14).

Aspirin's potential anti-proliferative effects on melanoma have not, until recently, been studied. Melanoma is the rarest type of skin cancer, accounting for only 2% of all cases (about 74,000 cases in the US in 2015), yet is the cause of the highest percentage of skin cancer related deaths (about 10,000). There have been relatively few clinical studies in which investigators have tried to identify any reduction in risk of melanoma incidence or mortality. The results from those studies so far, have been mixed. There are far fewer studies *in vitro* utilizing melanoma cell lines when compared to those for colorectal cancer, but there is some evidence that aspirin has anti-proliferative effects in these systems (15-17). Both COX-2 dependent and independent mechanisms have been suggested to be primarily responsible for inhibition of melanoma cell proliferation and progression including inhibition of Glutathione activity, in which elevated levels have been associated with neoplastic progression and drug resistance (17,18). Aspirin has also been reported to inhibit melanoma cell migration through the inhibition of the matrix-metaloprotease-2 (MMP-2) activity both *in vitro* and *in vivo* (19).

Indole-3-Carbinol as an Anti-cancer Agent

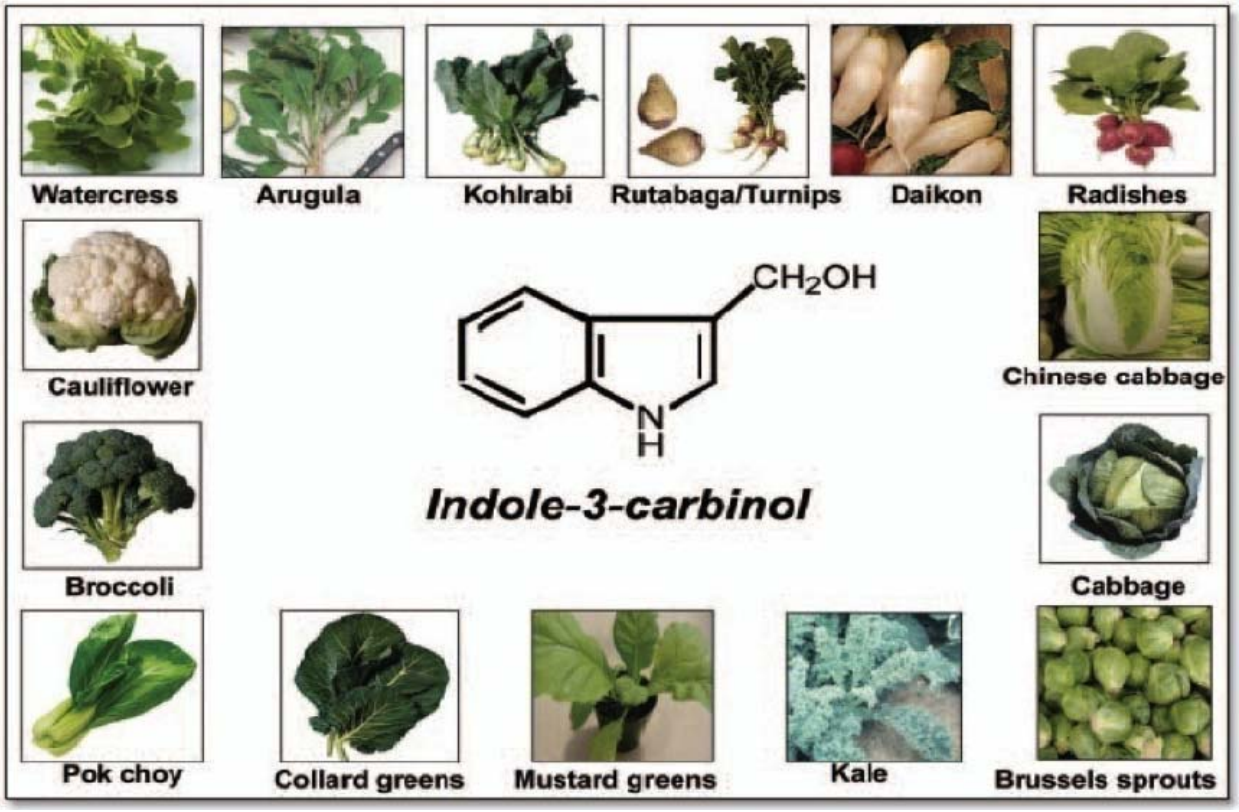
Indole-3-Carbinol (I3C) is a phytochemical that is generated from cruciferous vegetables of the *Brassica* genus such as Brussels sprouts, broccoli, and bok choy when they are crushed of cooked (20) (Figure 2) Many epidemiological studies over a span of decades have noted that an increased intake of these vegetables is correlated with, among other things, a decrease in rates of many cancers (21). When ingested, I3C is converted by stomach enzymes into its dimer 3,3' Diindolylmethane (DIM) as well as other metabolites. Through many studies it has been demonstrated that I3C and DIM, while both possessing anti-proliferative properties, act through distinct mechanisms in different cancer cell types including breast, skin, cervical, and many others (22,23).

In melanoma, I3C has been demonstrated to act through multiple mechanisms. Our lab has also discovered the one of the first specific targets of I3C, Neural Precursor Cell Expressed Developmentally Down-Regulated 4 (NEDD4-1) (publication in progress). NEDD4-1 is a ubiquitin ligase that tags the tumor suppressor Phosphatase and Tensin Homolog (PTEN) for destruction. By inhibiting the ligase activity of NEDD4-1, I3C stabilizes PTEN levels which counteracts the action of Phosphatidylinositol 3-kinase (PI3K), which is a critical enzyme for pro-proliferative and pro-survival signaling. Additionally, I3C works synergistically with ultraviolet B, to induce apoptosis in melanoma cells (24).

In Breast Cancer, the effects of I3C have been studied extensively. Recently, our lab discovered another specific target of I3C Human Neutrophil Elastase (HNE), an enzyme responsible for the production of hyperactive Cyclin E products via its ability to cleave the full-length form, as well as triggering the pro-survival CD40 pathway, both of which are drivers of proliferation in some advanced, non-hormone sensitive breast cancers I3C also has a profound effect on hormone-sensitive breast cancers. In MCF-7 cells, I3C caused an increase in the aryl hydrocarbon receptor (AhR) mediated degradation of Estrogen Receptor Alpha (ERα), which is at the node of multiple critical proliferative pathways (25). In addition, I3C is able to disrupt the transcription of the catalytic subunit of telomerase (hTERT) which is typically hyperactive in cancer cells and responsible, at least in part, for the immortalization process via extension of telomeres, and prevention of cellular senescence that normal, non-immortalized cells experience as they age (26).

Figure 2:

From (20): Indole-3-Carbinol is produced by cruciferous vegetables from the genus *Brassica*


Watercress
Arugula
Kohlrabi
Rutabaga/Turnips
Daikon
Radishes
Cauliflower
Chinese cabbage
CH2OH
N
H
Indole-3-carbinol
Broccoli
Cabbage
Pok choy
Collard greens
Mustard greens
Kale
Brussels sprouts

Microphthalmia-Associated Transcription Factor-M (MITF-M) in Melanoma

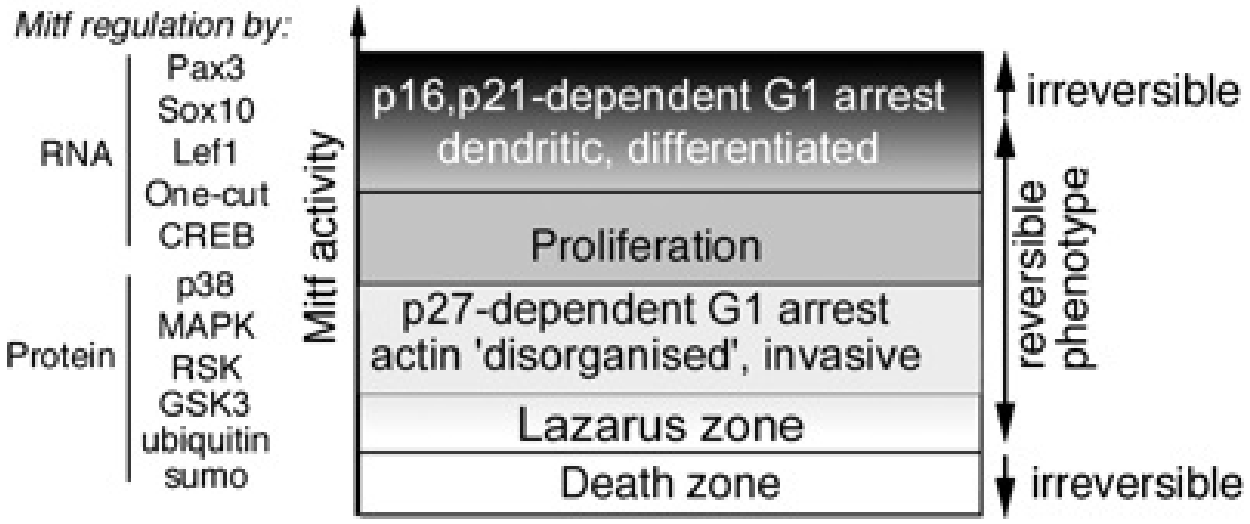
Micropthalmia-Assoiciated Transcription Factor-M (MITF) is a basic-helix-loop-helix-leucine-zipper transcription factor family whose family gives rise to nine different isoforms due to differential usage of alternative promoters and alternative splicing. Each isoform differs in their N-termini and their tissue-specific expression pattern (27), and can be expressed in multiple cell types including osteoclasts, natural killer cells, macrophages, and mast cells (28). These isoforms tend to be expressed in tissue specific expression patterns, with the MITF-M isoform typically expressing in melanocytes and melanoblasts. MITF-M often referred to as "master regulator" in both normal melanocyte development as well as melanoma progression. MITF mutations can cause Microphthalmia (an eye abnormality), Waardenburg Syndrome, sensorineural hearing impairment in humans and pigmentation defects in humans and other species (29,30). In melanocyctes, the pigment producing cells found in the deep layer of the epidermis, MITF-M is essential for development, by activating pigment producing genes such as dopachrome tautomerase and tyrosinase as well as the anti-apoptotic gene BCL-2 (31).

In melanoma, which are melanocyte derived tumors, MITF-M is a transcriptional activator that regulates genes associated with growth and proliferation and is considered a "lineage addiction" oncogenes since it has oncogenic effects yet is necessary for the survival of melanocytes (32). In primary melanoma tissue, MITF-M was found to be amplified in 10-20% of cases, and even higher in metastatic melanoma, and expression is negatively associated with five-year survival (29). MITF-M is an interesting gene to study because it acts as a molecular rheostat, lower levels of expression can lead to cellular senescence, while very high levels have been demonstrated to cause growth arrest. Intermediate to high levels promote progression through the cycle, cell survival, DNA repair, and cell migration (28,33,34) (Figure 3). As such, elevated levels of MITF-M can simultaneously inhibit invasiveness via regulation of matrix-metaloprotease proteins, and promote proliferation via negative regulation of the tumor suppressor Cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) and upregulation of Cyclin Dependent Kinase 2 (CDK2) (35), critical regulators of the G_1/S cell cycle transition. Given this, MITF-M represents an essential regulator of melanoma, and an important target whose regulation is critical to understand in both in basic research as well as clinical studies.

Figure 3:

From (34): **MITF acts as a "molecular rheostat"**

Low levels of MITF lead to invasive cells that are arrested in the G_1 phase. High levels lead to arrest driven by other factors, and a more differentiated phenotype.


Mitf regulation by:
RNA
Pax3
Sox10
Lef1
One-cut
CREB
Protein
p38
MAPK
RSK
GSK3
ubiquitin
sumo
Mitf activity
p16,p21-dependent G1 arrest
dendritic, differentiated
Proliferation
p27-dependent G1 arrest
actin 'disorganised', invasive
Lazarus zone
Death zone
irreversible
reversible phenotype
irreversible

Wnt/β-catenin Pathway Overview

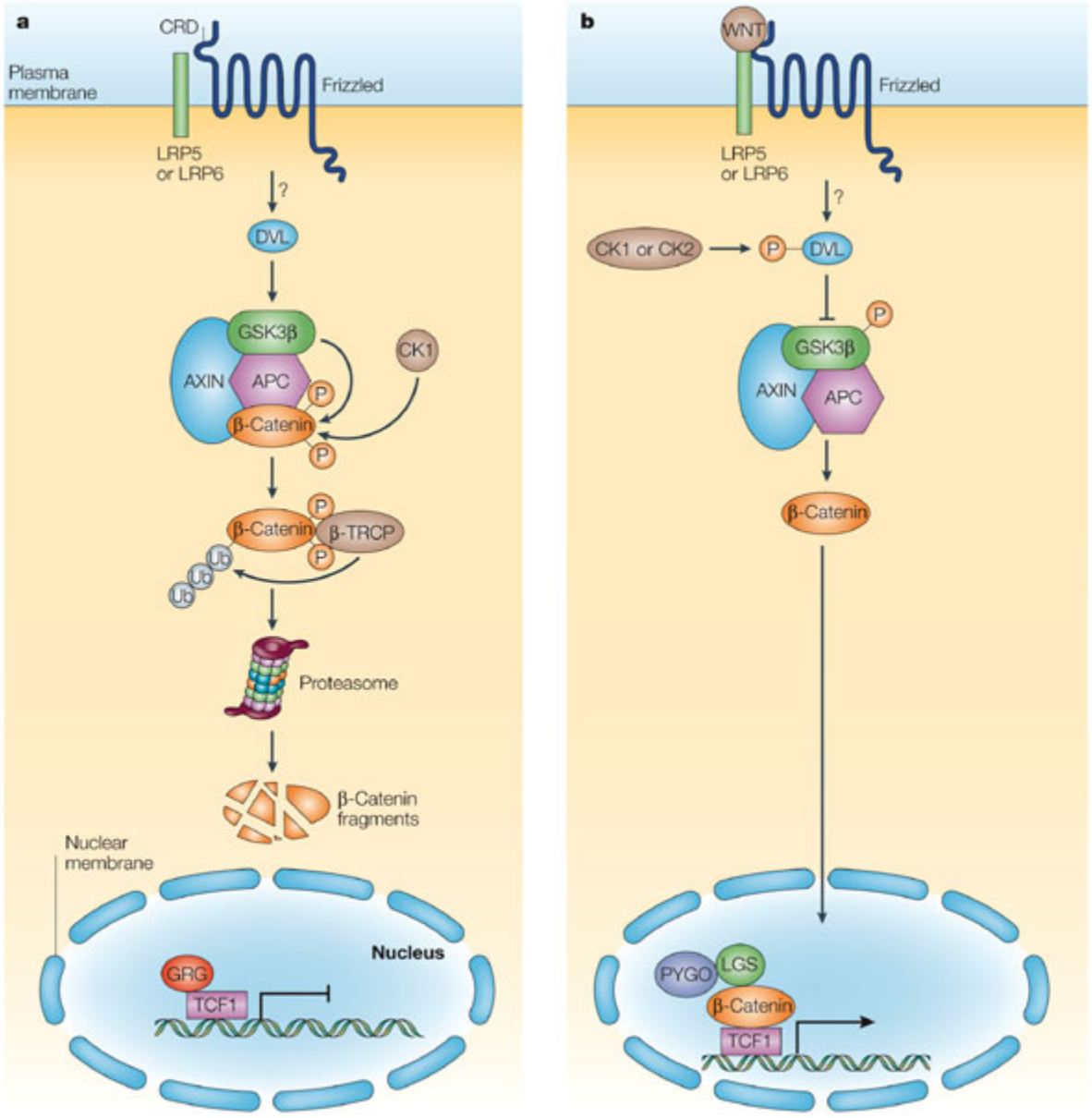
In normal cells Wnt/β-catenin signaling is important for development as well as important stem cell processes, and in normal cells plays a role in cell polarity, movement, proliferation, differentiation, survival and self-renewal (36). WNT's are a family of 19 secreted glycoproteins that can interact with the ten members of the Frizzled (FZD) family as well as other receptor tyrosine kinases (RTK's) to initiate signal transduction. In the canonical Wnt signaling pathway, most of these ligand/receptor interactions eventually converge on β-catenin to regulate downstream genes. Non-cannonical Wnt signaling pathways that do not involve β-catenin are less well understood and will not be explored in this thesis. In the canonical Wnt signaling pathway, when there are no Wnt protein molecules present, and the transcription factor β-catenin is targeted for phosphorylation and ultimate polyubiquitination and destruction by the "destruction complex" generally made up of Adenomatous polyposis coli (APC), Axin, Glycogen synthase kinase 3 beta (GSK3β), and Casein kinase 1 (CK1α). Normally, cytoplasmic β-catenin has the ability to translocate to the nucleus, form an activating complex with the TCF/LEF family of transcription factors, and activate Wnt target genes, but with decreased protein stability, there is a smaller pool of β-catenin which can activate downstream target genes, limiting Wnt signaling. When Wnt signaling is "on", Wnt family of proteins bind to Frizzled (Fzd) receptors and their co-receptors Low-density lipoprotein receptor related protein (LRP5/6) on the cell membrane. This complex recruits Axin to the cell membrane and away from the destruction complex. Without Axin to facilitate the phosphorylation by CSK3β and CK1α necessary to tag β-catenin for destruction, cytoplasmic pools of β-catenin increase, and as a result there is more available to form complexes with TCF/LEF transcription factors and activate target genes (37,38).

In many cancers, especially colorectal, disregulation of the Wnt/β-catenin pathway is often observed. In fact, an activating mutation in the Wnt/β-catenin is almost always the first step in the development of colorectal cancers (39). More than 80% of colorectal cancers have mutations in the *APC* gene inactivating it and its ability to associate with the rest of the destruction complex, which leads to the stabilization of cytoplasmic β-catenin. In colorectal cancers that do not have this mutation, about 50% have mutations in the *CTNNB1* (β-catenin) gene, altering key phosphorylation sites on the protein preventing it from being targeted for destruction (40,41).

In melanoma, the role of Wnt signaling is much less clear than in other malignancies. Some studies have implicated the aberrant activation of the Wnt/β-catenin pathway is oncogenic (42) and that blocking β-catenin expression in metastatic melanoma cell lines could induce apoptosis, inhibit proliferation, and migration (43). Others have demonstrated that elevated Wnt signaling and β-catenin expression correlates with better prognosis in patients (44,45).

Figure 4:

From (46): Overview of canonical Wnt signaling. When Wnt ligands bind the receptor-co-receptor complexes, Frizzled-LRP, resulting in inactivation of GSK3β by disheveled. Upon inactivation of GSK3β, β-catenin is not phosphorylated and targeted for destruction and is free to migrate to the nucleus where it can bind TCF/LEF transcription factors and activate downstream genes. The translocation of β-catenin into the nucleus depends on two proteins, pygopus homologue (PYGo) and legless homologue (LGS)



Nature Reviews | Immunology

Artemisinin and Artesunate derivatives as anti-cancer agents

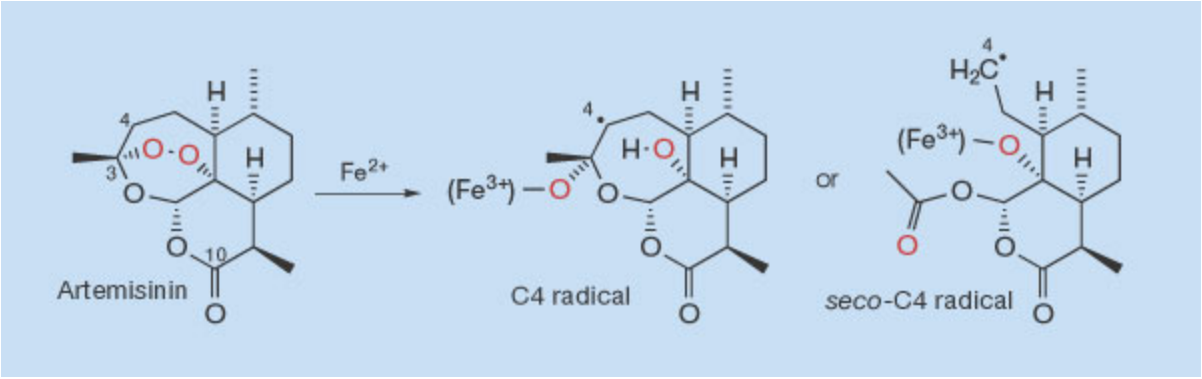
Artemisinin is a naturally occurring sequiterpene lactone originally isolated from the *Artemisia annua*, or sweet wormwood plant (47). For at least 2000 years, Artemisinin has been used to treat a wide variety of maladies including fever and hemorrhoids, and for the last 2 decades has been used as a potent anti-malarial therapy (48). Artemisinin's mechanism of action depends on the endoperoxide bridge within the molecule. Briefly, the endoperoxide bridge breaks open in the presence of heme-iron, generating free-radical reactive oxygen species (ROS) and other intermediates (49) (Figure 5). These intermediates form covalent bonds with malarial specific proteins and can lead to alkylation of specific malaria receptor proteins, inactivating them and interfering with cellular processes inside the parasite (50). These free radicals can also damage food vacuole membranes and lead to autolysis of the parasite (49). Interestingly, Artemisinin is able to specifically target malaria parasites and not the host cells because the parasites tend to upregulate intake of hemoglobin to utilize it as their primary source of amino acids, and as a result the parasites have an elevated level of iron in the cell when compared to normal cells.

While Artemisinin itself is effective against malaria, it has a short half-life *in vivo* and poor bioavailability. As a result several derivatives were developed to counter these drawbacks, these include; Dihydroartemisinin (DHA), Artemether, Artelinic acid, and Artesunic acid (Artesunate) all of which have been shown to be effective in treating different forms of the malaria parasite (49). Of these derivatives, Artesunate has been the most widely used derivative to treat malaria and does so at more effective doses than the parent molecule in combinations with other drugs (51).

Figure 5:

From (52) Artemisinin mechanism of action in Malaria.

The endoperoxide bridge of Artemisinin (red) is cleaved upon interaction with ferrous iron that comes from hemeoglobin which is in abundant supply in the neighboring red blood cells. Cleavage creates free radicals than can induce mitochondrial damage, activate caspases, and cause cell death in the parasite.


Artemisinin
Fe2+
(Fe3+)
C4 radical
or
H2C
(Fe3+)
seco-C4 radical

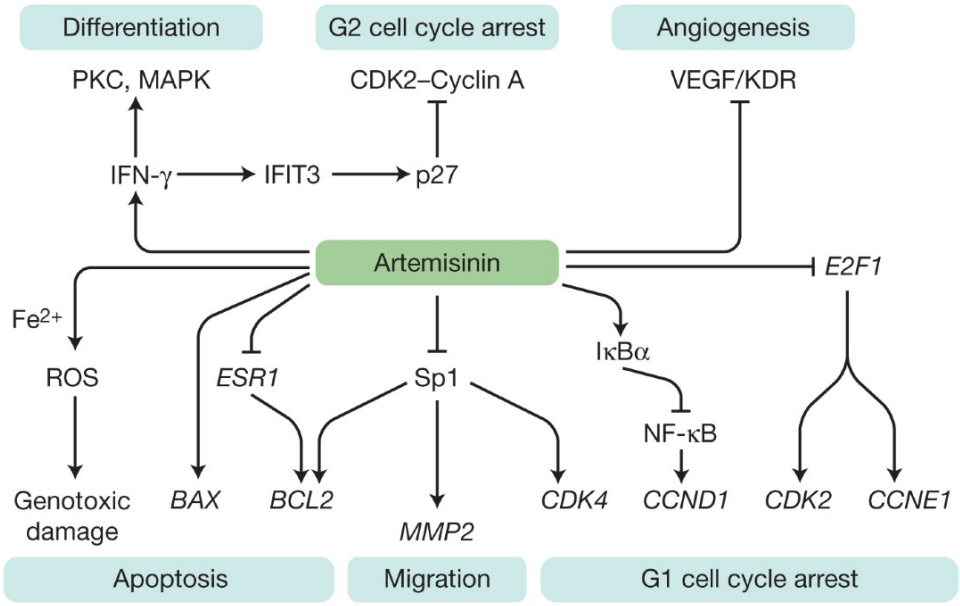
Artemisinin and its derivatives have been extremely useful anti-parasitic molecules for decades, as illustrated by the co-recipient of the 2015 Nobel Prize in Medicine, Youyou Tu, the woman who discovered Artemisinin. More recent evidence has demonstrated that Artemisinin and its derivatives have anti-cancer activities in multiple human cancer subtypes including leukemia, melanoma, breast, lung, prostate, pancreatic ovarian, and colon. Studies to determine potential Artemisinin mechanisms of action in cancer have demonstrated one similar to its anti-malarial mechanism. Many cancer cells have elevated iron when compared to the normal cells surrounding it (53) and this excess iron triggers the production of free radicals which can irreversible damage cellular DNA and proteins.

Even though it has been demonstrated that one mechanism of action in which Artemisinin slows cancer progression is through the generation of ROS species, several studies have demonstrated changes in expression of many oncogenes and tumor suppressor genes that cannot be accounted for by changes restricted to genes responsible for iron metabolism (54,55). The Firestone lab has demonstrated control of the Cyclin Dependent Kinase 2 and 4 (CDK2 and CDK4) in prostate cancer and endometrial cancer cells respectively by Artemisinin, as well as the E2 transcription factor (E2F) and Estrogen Receptor signaling in breast cancer (47,56-58). Other labs have demonstrated Artemisinin and its derivatives can induce cell cycle arrest, apoptosis through a decrease in anti-apoptotic genes *BCL2* and *BCL2L1* and increase in *BAX* and BAD, inhibition of angiogenesis through inhibition of vascular endothelial growth factor (VEGF), as well as effecting nuclear receptor activity (48,59,60). In addition Artemisinin can control cell migration in melanoma cells by decreasing the expression of matrix metalloproteinase 2 (MMP2) and $\alpha v\beta 3$ integrins (61). The anticancer effects of Artemisinin are summarized in Figure 6.

Figure 6:

From (48): The anticancer effects of Artemisinin.

Artemisinin has been demonstrated to slow the progression of multiple cancer types by exhibiting control of migration, apoptosis, angiogenesis, differentiation, and cell cycle through a variety of different pathways.



Molecular alterations induced in cancer cells leading to anticancer effects of artemisinin and related compounds

The Cell Cycle

The proliferation of the cell is governed by a complex process called "the cell cycle" which involves the activities of numerous proteins to function properly. Briefly, the cell cycle can be divided into 4 phases; Mitosis, Gap 1 (G_1), Synthesis Phase, and Gap 2 (G_2). In the G_1 phase, critical decisions are made whether the cell should exit the cell cycle all together or continue into the next phase. If there is DNA damage, the cell will not go past the restriction point that separates the G_1 and S phases. If there is not DNA damage the cell enters the S phase, in which the process of DNA duplication begins in preparation of eventual mitosis. Once the DNA is duplicated without damage, the cell enters the final phase before mitosis, the G_2 phase in which more preparations are made before the cell splits in M phase. After the typical mammalian cell spends a few hours in this phase, the process of mitosis commences in which the cell splits itself into two new cells. This process is very complex and has been studied for decades and will not be discussed in detail here. Each one of these steps is highly regulated to make sure there are no errors in the process.

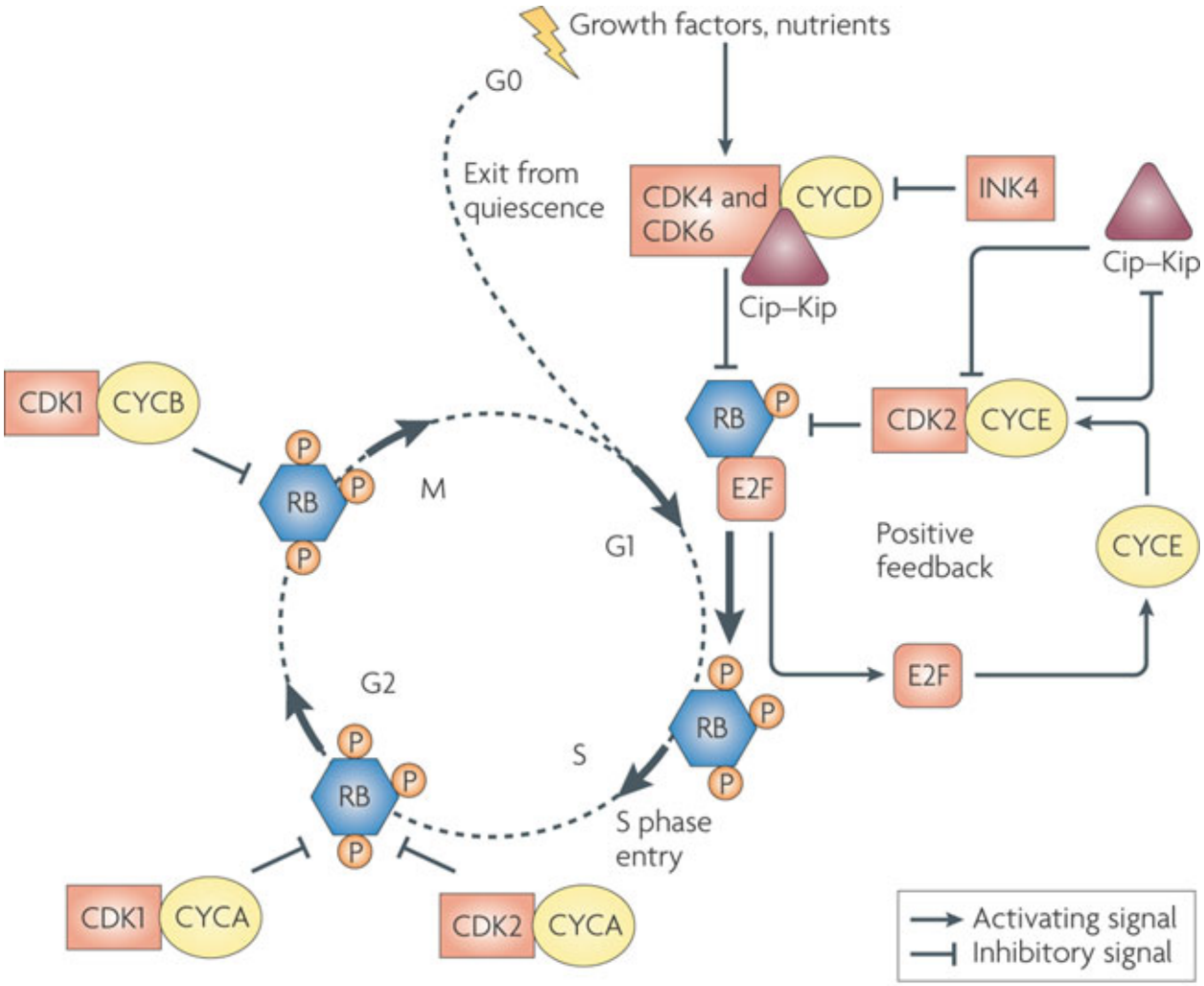
While there are many important proteins involved in the cell cycle, two of the key players are cyclins and cyclin dependent kinases (CDKs). Cyclins are named because their expression cycles with the phase of the cell, and CDKs are serine/threonine kinases that cannot act on their own. Cyclins regulate the activity of CDKs to promote phosphorylation of different targets, which activate those targets to drive the cell cycle. In the G_1 phase, D-type cyclins interact with CDKs 4 and 6 to promote phosphorylation of the rentinoblastoma protein (Rb). In the late G_1 phase, the expression of E-type cyclins rise and regulate CDK2, which hyperphosyphorylates Rb. Upon hyperphosphorylation, Rb releases the E2F family of transcription factors which allows them to regulate gene expression that will allow the cell to continue into the S phase. In the S-phase cyclin A and CDK1 and 2 work together to drive the cell through S phase and enter into the G_2 phase. Upon entry into G_2 cyclin B regulates CDK1 to push the cell into mitosis (62), this is summarized in Figure 7.

In all types of cancer, the normal regulation of the cell cycle is disrupted (63). Often, some of the drivers of the cell cycle are overexpressed or hyperactive, or some of the proteins whose function is to slow or stop the cycle at certain point are underexpressed or non-functional. Targeting these cell cycle components is one essential strategy in developing new, effective therapeutics.

Figure 7:

From (64): Overview of the mammalian cell cycle.

Cyclins and CDK's work together in each phase of the cell cycle to promote progression into the next phase. The Cip-Kip family, also known as p27, p21, and p57c inhibit the action of CDKs and act as the "brakes" in the normally controlled cell cycle. Without proper controls either through deletions or mutations, the cell could enter a quiescent state or progress through the cell cycle unregulated.



Nature Reviews | Drug Discovery

Cyclin A and the Cell Cycle

Cyclin A2 (Cyclin A) is overexpressed in many cancers and has been implicated to play a role in the epithelial-mesenchymal transition (65-67). It has recently been tagged as an effective prognostic marker for Estrogen Receptor (ER) positive breast cancer, whose levels are inversely correlated metastasis free survival, as well as disease free survival (68,69). It has also been associated with Tamoxifen resistance in ER positive breast cancers. Cyclin A is unique in that it is able to regulate the activity of two different CDKs, (CDK1 and CDK2). As a result, it has the capability to regulate different parts of the cell cycle. For decades, Cyclin A has been considered the "S phase cyclin" since its expression is highest in that phase (70). Recently though, investigators have begun to realize the role it plays outside of driving DNA synthesis. In the late G_1 phase, cyclin A levels and activity increase after cyclin E/CDK2 to *activate* DNA synthesis and bring the cells into S phase from late G_1 by inducing centrosome duplication, among other functions. In addition to induction of replication, the cyclin A/CDK2 complex stops pre-replication complex assembly that was promoted by the CyclinE/CDK2 complex, by phosphorylation and inactivating Cell Division Cycle 6 (Cdc6), a crucial regulator of initiation and initiates DNA synthesis (71). Without either cyclin, the cell would arrest at the G_1/S transition. In the G_2/M transition, cyclin A has been implicated in stabilizing cyclin B, and its ectopic injection into *Xenopus* oocytes or mammalian cells stimulates entry into M phase, though its exact role is still unclear (72). Knockdown of cyclin A has been demonstrated to arrest cells in G_2 (73,74).

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Chapter I

Cooperative Anti-Proliferative Response by Aspirin and Indole-3-Carbinol Targeted Cell Signaling Pathways Disrupt Micropthalmia Associated Transcription Factor Gene Expression and Promoter Activity in Human Melanoma Cells

Abstract

Anti-proliferative signaling of combinations of the nonsteroidal anti-inflammatory drug acetylsalicylic acid (aspirin) and indole-3-carbinol (I3C), a natural indolecarbinol compound derived from the breakdown of glucobrassicin produced in cruciferous vegetables, was investigated in human melanoma cells. Melanoma cell lines with distinct mutational profiles were sensitive to different extents to the anti-proliferative response of aspirin, with oncogenic BRAF-expressing G361 cells and wild type BRAF-expressing SK-MEL-30 cells being the most responsive. I3C triggered a strong proliferative arrest of G361 melanoma cells, and caused only a modest decreased proliferation of SK-MEL-30 cells. In both cell lines, combinations of aspirin and I3C cooperatively arrested cell proliferation and induced a G_1 cell cycle arrest, and nearly ablated protein and transcript levels of the melanocyte master regulator Micropthalmia Associated Transcription Factor isoform M (MITF-M). In melanoma cells transfected with a -333/+120 bp MITF-M promoter-luciferase reporter plasmid, treatment with aspirin and I3C cooperatively disrupted MITF-M promoter activity, which accounted for the loss of MITF-M gene products. Mutational analysis revealed that the aspirin required the Lymphoid Enhancer-binding Factor 1 (LEF1) binding site, whereas, I3C required the BRN2 transcription factor binding site to mediate their combined and individual effects on MITF-M promoter activity. Consistent with LEF1 being a downsteam effector of Wnt signaling, aspirin, but not I3C, downregulated protein levels of the Wnt co-receptor LDL receptor-related protein-6 and β-catenin and up-regulated the β-catenin destruction complex component Axin. Taken together, our results demonstrate that aspirin-regulated Wnt signaling and I3C targeted MEK/MAPK cascades converge on the MITF-M promoter to cooperatively disrupt MITF-M expression and melanoma cell proliferation.

Introduction

Human melanomas, which arise from melanocytes of neuro-ectoderm origin, are the most aggressive form of malignant skin cancers, and can be categorized by distinct mutational profiles that determine their corresponding cellular phenotypes, proliferative capabilities, and therapeutic options (1). For example, in approximately 60% of melanomas, mutations within the *BRAF* gene result in expression of a constitutively active Ser/Thr protein kinase with oncogenic properties (2). Signaling by oncogenic BRAF leads to constitutive activation of MEK and Erk/MAPK, and in recent years new therapeutic approaches have been developed that target specific components of this cellular cascade, such as the oncogenic BRAF inhibitors Vemurafenib (3) and Dabrafenib (4) as well as the MEK inhibitor Trametinib (5). A critical downstream effect of the oncogenic BRAF pathway is enhanced levels of expressed Microphthalmia Associated Transcription Factor isoform M (MITF-M), a basic helix-loop-helix leucine zipper transcription factor highly involved with melanocyte lineage cell processes (6). Through its transcriptionally regulated gene products, MITF-M plays an essential role in cell survival, morphology, migration, differentiation and proliferation of melanocytes and has a complex role in the malignant transformation, progression, proliferation and metastasis of melanoma (7,8). The levels of MITF-M in the context of the mutational status is considered to be a critical factor in determining the efficacy and treatment outcomes of a given melanoma therapy and probability of relapse of the disease (9). Therefore, an essential foundation to develop new pre-clinical therapeutic strategies for melanoma is to assess effects on MITF-M, although a mechanistic understanding of the regulation of MITF-M gene expression by potential anti-cancer agents is not well understood.

Dietary phytochemicals and their synthetic analogues represent an intriguing group of compounds with the potential to regulate melanoma tumor growth and spread of malignancies through multiple cellular pathways with minimal side effects (10,11). One such molecule is Indole-3-Carbinol (I3C), a naturally occurring compound derived from glucobrassicin made in cruciferous vegetables of the *Brassica* genus, such as broccoli, cauliflower, and Brussels sprouts (12). I3C has been shown to trigger anti-proliferative and pro-apoptotic properties *in vivo* and in a variety of cultured human cancer cells by controlling specific transcriptional, enzymatic, metabolic, and growth factor and hormone-dependent cell signaling processes (13-16). In clinical trials, I3C has been demonstrated to be tolerated well with minimal side effects (17). From a mechanistic viewpoint, we have established that the presence of specific I3C target proteins expressed in human cancer cells, such as Human Neutrophil Elastase (18) in breast cancer and the Neural precursor cell Expressed Developmentally Down-regulated 4 (NEDD4-1) a ubiquitin ligase whose function is critical in a subset of melanoma genotypes (19). Inhibition of these target proteins' activities, and possibly others, mediates the efficacy by which I3C selectively stimulates distinct anti-proliferative signaling cascades (20,21). Other studies have shown that I3C treatment increased sensitivity to UV-induced apoptosis and enhance cytotoxic responses in human melanoma (22,23) and squamous cell (24), respectively. Also, ectopic application of I3C directly inhibits skin tumor formation in mouse models (25). We have observed that human melanoma cells with distinct mutational profiles are sensitive to the anti-proliferative effects of I3C (19), suggesting that this natural phytochemical could provide one component of a combinational therapy for human melanomas.

Another molecule that was originally discovered from plants that has anti-cancer properties and is safely ingested by humans is acetylsalicylic acid (aspirin). Daily intake of aspirin has been shown to significantly reduce overall tumor burden in cancer patients (26). For example, long-term ingestion of low doses of aspirin reduced the incidence of melanoma in women (27), and other clinical studies showed lower rates of colorectal, esophageal, pancreatic, and lung cancer in patients taking aspirin (28,29). The cellular mechanism of aspirin action accounting for its anti-cancer effects has been investigated in several cancer cell model systems including melanoma cells (30,31). Recent studies have pointed to aspirin's ability to inhibit Cyclooxygenase 1 and 2 (COX-1/2) enzymatic activity as playing a role in the anti-proliferative effects of aspirin. In addition, aspirin treatment leads to the disruption of Nuclear Factor Kappa-light-chain enhancer of activated B cells signaling, and activation of apoptotic pathways (32-34). Despite these observations, aspirin's mechanisms of action outside of COX-1/2 inhibition have not been studied in depth in melanoma and nothing is known about the potential effects of aspirin on MITF-M levels.

Multiple cell signaling pathways converge on the MITF-M promoter and protein, suggesting that this critical regulator of melanoma proliferation can be used to assess potential effectiveness at a pre-clinical level of newly developed therapeutic strategies for melanoma. In this study, we now demonstrate that a combination of I3C and aspirin cooperatively arrest the proliferation and induce a G_1 cell cycle arrest of human melanoma cells and nearly ablate MITF-M gene expression. Aspirin and I3C anti-proliferative signaling converges on the MITF-M promoter through the LEF1 binding site, a downstream effector of the Wnt pathway, and the BRN2 binding site, a downstream effector of the BRAF-MEK-MAPK signaling cascade. Our study expands on the limited mechanistic understanding on aspirin's mode of action in melanoma cells and regulation of MITF-M expression, as well as implicating at a pre-clinical level a combination of aspirin and I3C as a new potential therapeutic strategy for melanoma.

Materials and Methods

Reagents and cell culture

Indole-3-Carbinol was purchased from Sigma-Aldrich (St. Louis, MO), Acetylsalicylic acid (aspirin) was purchased from Fisher Scientific (Waltham, MA). All cell lines were obtained from American Type Culture Collection (Manassas, Virginia). G361 cells were cultured in McCoy's 5A Modified medium (Lonza, Walkersville, MD) supplemented with 10% Fetal Bovine Serum (Gemini, Elizabeth, NJ), 2mM *L*-glutamine (Sigma-Aldrich), 50 U/mL penicillin, and 50 U/mL streptomycin (Sigma). SK-MEL-30 cells were cultured in Dulbecco's Modified Eagle's Medium (Lonza) supplemented with 10% Fetal Bovine Serum, 2mM *L*-glutamine, 1X MEM-NEAA (Life Technologies, Carlsbad, CA) 50 U/ml penicillin, and 50 U/ml streptomycin. Cells were grown in a humidified chamber at 37^{o} containing 5.0% CO_2. 1000X solutions of I3C and aspirin were made in dimethyl sulfoxide (DMSO) then diluted to 1X in media before being added to the plate.

Cell proliferation assay

Cells were plated onto 24-well tissue culture plates (Nunc, Roskilde Denmark) at 70% confluency and treated as indicated in triplicate with DMSO vehicle, aspirin alone, I3C alone, or combinations of the two, for 48 hours. Inhibition of proliferation was measured using the Dojindo Cell Counting Kit-8 (Rockville, Maryland) as per manufacturer's instructions. Briefly, 50µL of the CCK-8 solution was added to each well along with 450µL of full media and incubated for 2.5 hours. Absorbance was read at 450nm and percent inhibition was calculated by standardizing the average of each treatment triplicate to the average value of the vehicle control.

Flow cytometry

Cells were plated onto six-well tissue culture plates at 70% confluency and treated as indicated in triplicate with the medium changed every 24 hours. Following the treatment the cells were washed once with phosphate buffered saline (PBS) (Lonza) and harvested with 1mL of PBS. Cells were then lysed in 300µL of DNA staining solution (0.5mg/mL propidium iodide, 0.1% sodium citrate, and 0.05% Triton X- 100). Nuclear fluorescence of wavelength more than 585nm was measured on a Beckman-Coulter EPICS XL instrument with laser output adjusted to deliver 15 megawatts at 488nm. For each sample 10,000 nuclei were analyzed and the percentage of cells in G_1, S, G_2/M phase of the cell cycle was determined by analysis with Multicycle provided by Phoenix Flow Systems in the Cancer Research Laboratory, Flow Cytometry Facility of the University of California, Berkeley.

Western blots

After the indicated treatments, G361 and SK-MEL30 cells were harvested then pelleted by centrifugation. The cells were then re-suspended in radio immunoprecipitation buffer (50mM Tris pH. 8.0, 150 mM NaCl, 0.1% SDS, 0.1% NP-40, and 0.5% Sodium Deoxycholate) containing protease inhibitors (50 µg/mL phenylmethysulfonyl fluoride, 10 µg/mL aprotinin, 5 µg/mL leupeptin, 0.1 µg/mL NaF, and 10 µg/mL β-glycerophosphate). Western blotting was carried out as previously described (35), and proteins were visualized using Enhanced

Chemiluminescence Lightning reagent (GE Healthcare, Piscataway, NJ) on nitrocellulose membranes. Mouse anti-MITF antibodies (Thermo-Fisher, #MA5-14146) was diluted 1:200 in wash buffer. Rabbit anti-CDK2 (Santa Cruz Biotechnology, Inc., Santa Cruz CA, #sc-163) was diluted 1:500 in wash buffer. Mouse anti-HSP-90 (BD Transduction Labs, San Jose, CA, #610418) was diluted 1:1000 in wash buffer.

Reverse transcription-polymerase chain reaction

Cells were treated as indicated, harvested and pelleted by centrifugation. Total RNA was isolated with Trizol Reagent (Invitrogen, San Diego CA) according to manufacturer's protocol. Total RNA and RNA quality was measured using a NanoDrop. Total RNA was subjected to reverse transcription using M-MLV Reverse Transcriptase (Invitrogen), random hexamers, deoxynucleotide triphosphates, and RNAse inhibitor (Invitrogen). cDNA (0.5 µg) was then amplified using the following primers: MITF forward, 5'- ATGCTGGAAATGCTAGAATAT-3' and reverse, 5'-CAATCAGGTTGTGATTGTCC-3'; CDK-2 forward, 5'-CCAGTACTGCCATCCGAGAG -3' and reverse, 5'- CGGCGAGTCACCATCTCAGC -3'; GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'. PCR conditions were as follows: 30s at 94^{o}, 30s at 55^{o} for GAPDH 57^{o} for MITF, and 30s at 72^{o} for 26 cycles. Products were fractionated on a 1.1% agarose gel containing 0.01% Ethidium Bromide and visualized by a UV transilluminator.

Transfections and luciferase reporter plasmid assays

The pMITF-333/+120-pGL2, MITF promoter-luciferase reporter plasmid was a kind gift from Dr. Richard Marais (Cancer Research UK Centre of Cell and Molecular Biology, London, United Kingdom). G361 and SK-MEL30 cells were grown to 70% confluence in six-well plates (Nunc) and transfected with 2 µg/well DNA of the indicated plasmid construct. Transfections were performed using either Superfect (Qiagen) for G361 cells or Fugene6 (Promega, Madison, WI) for SK-MEL-30 cells as per manufacturer's instructions. A ratio of 2 Superfect:1µg DNA was used for transfecting G361 cells and 4 Fugene6:1µg DNA for SK-MEL-30 cells. Cells were treated 24 hours post-transfection as indicated for 48 hours. Cells were then harvested and lysed and relative luciferase activity was evaluated using the Luciferase assay kit (Promega). Relative luciferase activities were normalized to protein input as determined by the Bradford protein assay. These results were verified by three independent experiments performed with triplicate samples of each treatment.

Generation of MITF-M promoter mutations

Mutations were generating utilizing the pMITF-333/+120-pGL2 as a template. pMITF-333/+120-pGL2-ΔLEF1(ΔLEF1) was generated using the following primers: LEF1 forward, 5'-GACAGTGAGTTTGACTTTGGCAGCTCGTCACTTAA -3' LEF1 reverse 5'-TTAAGTGACGAGCTGCCAAAGTCAAACTCACTGTC -3'. pMITF-333/+120-pGL2-ΔBRN2(ΔBRN2) was generated using the following primers: BRN2 forward, 5'-TACATGCATAACTAGCGAGCTTAGGTTATTATAAGC -3' BRN2 reverse, 5'-GCTTATATTAACCTAAGCTCGCTAGTTATGCATGTA -3'. The following PCR conditions were utilized; -ΔLEF1- 2 min at 95^{o}, 30 s at 95^{o}, 30 s at 59^{o}, 8 min at 72^{o}, for 30 cycles, -ΔBRN2- 30 s hotstart at 95^{o}, 30 s at 95^{o}, 1 min at 55^{o}, 8 min at 68^{o}, for 16 cycles. -ΔLEF1-ΔBRN2 Double mutant was made by utilizing the -ΔBRN2 mutant as a template and inserting

the LEF1 mutation as described above. Mutagenesis was performed using QuickChange II kit (Aligent) per the manufacturer's instructions. PCR products were extracted and purified using QIAquick Gel Extraction Kit (Qiagen). Sequence was confirmed by automated DNA sequencing (University of California Berkeley Sequencing Facility).

Measurement of PGE_2 levels by enzyme-linked immunosorbent assay

Cells were grown to about 70% confluency in 6-well plates, and then treated for 48 hours as indicated in triplicate, changing media every 24 hours. Media was extracted from sample and PGE_2 levels were measured using the Prostaglandin E_2 ELISA Kit – Monoclonal (Cayman Chemical, Ann Arbor Michigan) as per manufacturer's instructions. After 90 minutes of development, absorbance was measured at 410nm.

Results

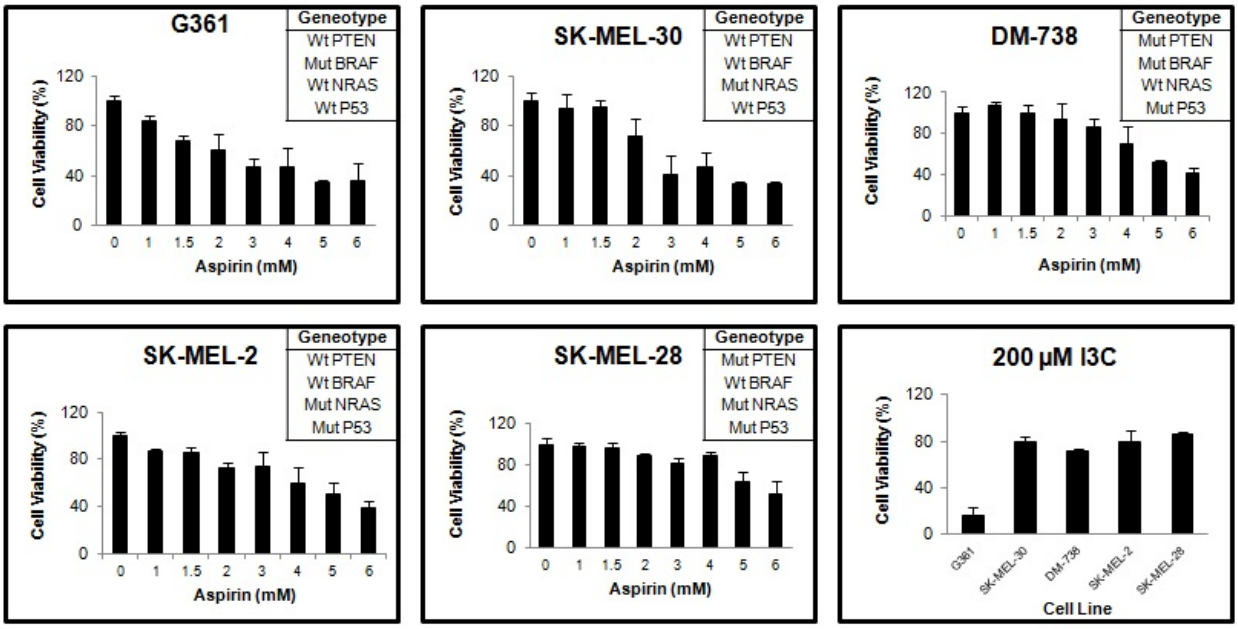
Anti-proliferative effects of aspirin and I3C in human melanoma cells that display distinct mutational profiles.

Human melanoma can be categorized by their mutational profiles, including expression of oncogenic forms of BRAF and NRAS, and mutations in tumor suppressor proteins such as PTEN and p53, which determine the corresponding cellular phenotypes, proliferative capabilities and therapeutic options (36). Therefore, to initially examine the potential anti-proliferative effects of aspirin, five different human melanoma cell lines (G361, SK-MEL-30, DM-738, SK-MEL-2, and SK-MEL-28 cells) were treated with increasing concentrations of aspirin, up to 6 mM, for 48 hours and the cell viability determined using a CCK-8 assay. Cells treated with only the vehicle control represent the 100% cell viability level for each cell line. As shown in Fig. 1. in all cell lines tested, aspirin caused a dose dependent decrease in total cell number compared to the vehicle control, although the efficacy of this anti-proliferative response differed somewhat between each of the tested melanoma cell lines. G361 and SK-MEL-30 cells were the most sensitive to aspirin treatment, with the half-maximal response occurring at approximately 3.0 mM aspirin (Fig 1), whereas, the other tested melanoma cell lines required higher concentrations of aspirin to induce a less pronounced anti-proliferative effect. There was no strong correlation between the overall mutagenic profiles and sensitively to aspirin, although the most sensitive melanoma cell lines express wild type forms of PTEN and p53. In a generally similar experiment, the anti-proliferative effects of 48-hour treatment with 200 μM I3C was assessed in each of the melanoma cell lines. G361 melanoma cells were the most sensitive cell line to the anti-proliferative effects of I3C, whereas, the other four cell lines were partially sensitive to the anti-proliferative effects of I3C (Fig 1, lower right panel). Therefore, because of their differential responsiveness to the anti-proliferative effects of aspirin and I3C and their distinct mutagenic profiles, G361 and SK-MEL-30 cells were used and compared throughout our study.

Figure 1

Effects of aspirin and I3C on the proliferation of melanoma cell lines with distinct mutational profiles.

Human melanoma cell lines (G361, SK-MEL30, DM-738, SK-MEL-2, and SK-MEL-28) displaying different genotypes (see panel insets) were treated with the indicated concentrations of aspirin for 48 hours or with 200 μM I3C for 48 hours (lower right panel). Cell proliferation was measured using a CCK-8 assay relative to the vehicle control. The results show the mean of three independent experiments.


G361
Geneotype
Wt PTEN
Mut BRAF
Wt NRAS
Wt P53
Cell Viability (%)
120
80
40
0
0 1 1.5 2 3 4 5 6
Aspirin (mM)
SK-MEL-30
Geneotype
Wt PTEN
Wt BRAF
Mut NRAS
Wt P53
Cell Viability (%)
120
80
40
0
0 1 1.5 2 3 4 5 6
Aspirin (mM)
DM-738
Geneotype
Mut PTEN
Mut BRAF
Wt NRAS
Mut P53
Cell Viability (%)
120
80
40
0
0 1 1.5 2 3 4 5 6
Aspirin (mM)
SK-MEL-2
Geneotype
Wt PTEN
Wt BRAF
Mut NRAS
Mut P53
Cell Viability (%)
120
80
40
0
0 1 1.5 2 3 4 5 6
Aspirin (mM)
SK-MEL-28
Geneotype
Mut PTEN
Wt BRAF
Mut NRAS
Mut P53
Cell Viability (%)
120
80
40
0
0 1 1.5 2 3 4 5 6
Aspirin (mM)
200 µM I3C
Cell Viability (%)
120
80
40
0
G361
SK-MEL-30
DM-738
SK-MEL-2
SK-MEL-28
Cell Line

Combinational effects of aspirin and I3C on the inhibition of melanoma cell proliferation and downregulation of MITF-M levels.

G361 and SK-MEL-30 cells were treated with aspirin or I3C alone or with combinations of sub-maximal or maximal concentrations of both compounds, and relative cell viability was analyzed using the CCK-8 assay. As shown in Fig 2A, 48 hour treatment of cells with increasing concentration of aspirin or I3C alone dose-dependently decreased cell viability. Interestingly, when each cell line was treated with sub-maximal combinations of both compounds, a synergistic effect on cell viability was observed. For example, compared to the vehicle control treated cells, treatment of G361 cells with 150 μM I3C treatment caused a 29% decrease in cell viability, and treatment with 1.0 mM aspirin resulted in a 21% decrease in cell viability. Treatment of G361 cells with a combination of 150 μM I3C and 1.0 mM aspirin triggered a 97% decrease in cell viability, an effect significantly greater than what would be expected from an additive effect of each compound. In SK-MEL-30 cells, the synergistic effect of aspirin and I3C combinations was less pronounced compared to the G361 melanoma cells. For both melanoma cell lines, other concentration combinations of aspirin and I3C acted cooperatively to inhibit melanoma cell proliferation (Fig 2A), suggesting that aspirin and I3C likely act through distinct cellular anti-proliferative pathways, with perhaps common downstream cellular targets.

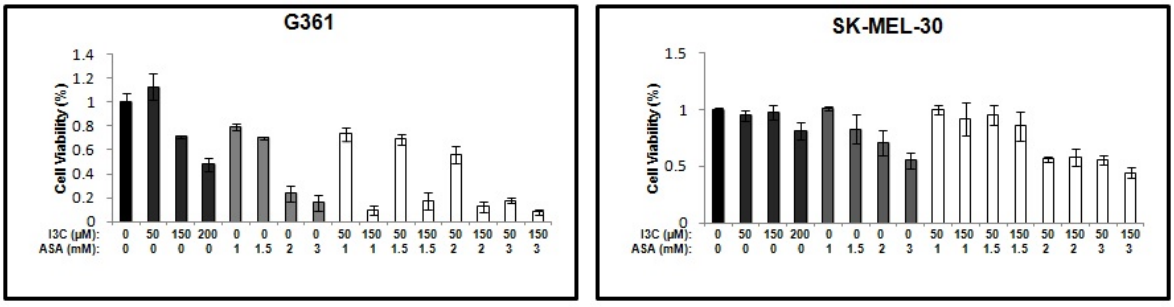
One potential downstream target of aspirin and I3C anti-proliferative signaling is MITF-M because this transcriptional regulator activates several critical melanoma cell pathways by regulating expression of genes directly involved in cell cycle progression, cell migration, and inhibition of apoptosis (37). Therefore, the effects of 48-hour treatments with combinations of aspirin and/or I3C on MITF-M protein levels were examined in both G361 and SK-MEL-30 human melanoma cells by western blot analysis. As shown in Figure 2B, in both melanoma cell lines, aspirin treatment had on only a modest effect on MITF-M protein levels compared to vehicle control treated cells. In contrast, I3C strongly downregulated MITF-M protein levels in G361 cells but had little effect on MITF-M levels in the SK-MEL-30 cells. Similar to effects on cell proliferation, treatment with sub-maximal combinations of both aspirin and I3C strongly downregulated MITF-M protein levels in both melanoma cells well beyond the effects of observed in cells treated with either compound alone (Fig 2B). This synergistic downregulation of MITF-M protein levels was first observed in G361 cells treated a combination of 1.0 mM aspirin and 50 μM I3C. Densitometry analysis demonstrated that I3C and aspirin treatment alone only reduced MITF-M levels by 7% and 2% respectively, whereas, treatment with the combination reduced MITF-M levels by 28%. This cooperative effect was even more pronounced upon treatment with 50 μM I3C and 1.5 mM aspirin, in which the individual treatments reduced protein levels by 7% and 20% respectively, but the combination nearly ablated the production of MITF-M protein. Similar effects were observed with the SK-MEL-30 cells under the same treatment conditions with the first observable synergistic effect on MITF-M protein levels was found upon treatment with a combination of 50 μM I3C and 2.0 mM aspirin. Treatment with a combination of 3.0 mM aspirin and 150 μM I3C strongly disrupt production of MITF-M protein. Taken together, these results demonstrate that compared to the effects of each compound alone, a combination of aspirin and I3C displays a significantly more potent anti-proliferative response including the loss of MITF-M protein production.

Figure 2

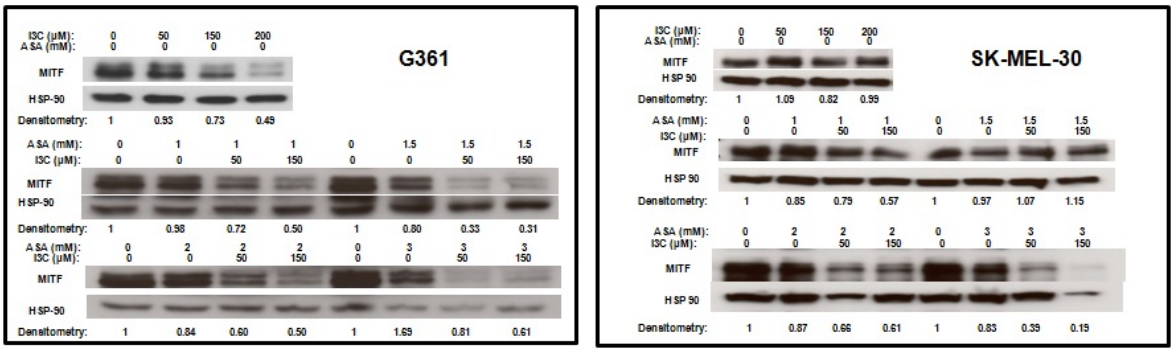
Combinational effects of aspirin and I3C on melanoma cell proliferation and production of MITF-M protein.

(A) G361 cells (left panel) and SK-MEL-30 cells (right panel) were treated with the indicated concentrations of aspirin (ASA) or I3C for 48 hours and cell proliferation was measured using a CCK-8 assay relative to vehicle control treated cells. (B) G361 cells (left panel) and SK-MEL-30 cells (right panel) were treated with the indicated concentrations of aspirin (ASA) or I3C for 48 hours and the levels of MITF-M protein were analyzed by western blots. The relative levels of MITF-M protein under each condition were measured by densitometry of the western blots and normalized to the HSP-90 loading control protein.

Fig 2A.


G361
Cell Viability (%)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
ASA (mM): 0 0 0 0 1 1.5 2 3 1 1 1.5 1.5 2 2 3 3
SK-MEL-30
Cell Viability (%)
1.5
1
0.5
0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
ASA (mM): 0 0 0 0 1 1.5 2 3 1 1 1.5 1.5 2 2 3 3

B.


I3C (μM): 0 50 150 200
ASA (mM): 0 0 0 0
MITF
HSP-90
Densitometry: 1 0.93 0.73 0.49
G361
ASA (mM): 0 1 1 1 0 1.5 1.5 1.5
I3C (μM): 0 0 50 150 0 0 50 150
MITF
HSP-90
Densitometry: 1 0.98 0.72 0.50 1 0.80 0.33 0.31
ASA (mM): 0 2 2 2 0 3 3 3
I3C (μM): 0 0 50 150 0 0 50 150
MITF
HSP-90
Densitometry: 1 0.84 0.60 0.50 1 1.69 0.81 0.61
I3C (μM): 0 50 150 200
ASA (mM): 0 0 0 0
MITF
HSP 90
Densitometry: 1 1.09 0.82 0.99
SK-MEL-30
ASA (mM): 0 1 1 1 0 1.5 1.5 1.5
I3C (μM): 0 0 50 150 0 0 50 150
MITF
HSP 90
Densitometry: 1 0.85 0.79 0.57 1 0.97 1.07 1.15
ASA (mM): 0 2 2 2 0 3 3 3
I3C (μM): 0 0 50 150 0 0 50 150
MITF
HSP 90
Densitometry: 1 0.87 0.66 0.61 1 0.83 0.39 0.19

Combinations of aspirin and I3C disrupt CDK2 expression and induce a cell cycle arrest in human melanoma cells.

MITF-M binds to the promoters and stimulates transcription of several cell cycle genes to promote melanoma cell proliferation (38). One such critical cell cycle gene target of MITF-M transcriptional activity is Cyclin Dependent Kinase 2 (CDK2), which contains an MITF-M DNA binding site (CATGTG) in its promoter at -1315 base pairs upstream of the transcriptional start site (39) (see Fig 3A diagram). Therefore, to functionally test the downstream effects of aspirin and I3C downregulation of MITF-M protein levels, the effects of 48-hour treatments of G361 cells with combinations of aspirin and/or I3C on CDK2 transcript levels was examined by RT-PCR analysis. As shown in Figure 3A, CDK2 transcript levels were modestly attenuated by treatment with I3C or aspirin in a dose dependent manner. When the cells were treated with combinations of the two compounds, a synergistic decrease of CDK2 mRNA levels was observed that generally correlated in a dose-dependent manner to the cooperative effect on MITF-M protein levels. Expression of CDK2 transcripts was almost completely abolished upon treatment with 3.0 mM ASA and either 50 μM or 150 μM I3C (Fig 3A), two concentration combinations that ablated MITF-M protein production in G361 cells (Fig 2B, lower right gels of left panel).

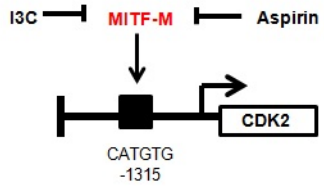
The loss of MITF-M protein levels and downregulation of its CDK2 target gene predicts that combinations of aspirin and I3C should strongly induce a cell cycle arrest of G361 and SK-MEL-30 melanoma cells. To test this possibility, both cell lines were initially treated for 48 hours with indicated concentrations of aspirin and I3C alone, and DNA content determined by flow cytometry analysis of propidium iodide stained nuclei. As shown in figure 3B, consistent with a G_1 cell cycle arrest, treatment of both melanoma cell lines with either aspirin or I3C dose-dependently induced an increase in G_1 phase DNA content with maximum effects observed at 3.0 mM aspirin and 200 μM I3C, respectively. Similar to effects on MITF-M levels, G361 melanoma cells displayed a stronger G_1 cell cycle arrest compared to the SK-MEL-30 melanoma cells. To assess the potential synergistic effects of aspirin and I3C, cells were treated for 48 hours with different concentration combinations of these compounds and DNA content analyzed by flow cytometry analysis of propidium iodide stained nuclei. At all of the combinations of sub-maximal concentrations of each compound, treatment with aspirin and I3C synergistically induced a G_1 cell cycle arrest, compared to cells treated with the compounds alone (Fig 3C, left panel). The combination of 3.0 mM aspirin and 150 μM I3C, both maximal concentrations, strongly induced a G_1 cell cycle arrest that was only slightly greater than the effects of each compound alone. A generally similar effect was observed with the SK-MEL-30 melanoma cells, although the final percentage of the cell population arrested with a G_1 DNA content was less than observed with the G361 cells. Taken together, these observations indicate that combinations of aspirin and I3C effectively downregulate MITF-M protein levels, causing the loss of CDK2 expression, resulting in a G_1 cell cycle arrest of the melanoma cells.

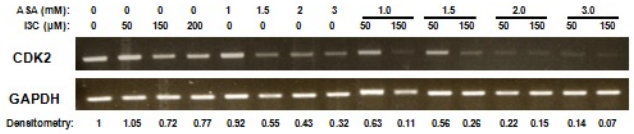
Figure 3

Aspirin and I3C regulation of melanoma cell cycle progression and expression of CDK2

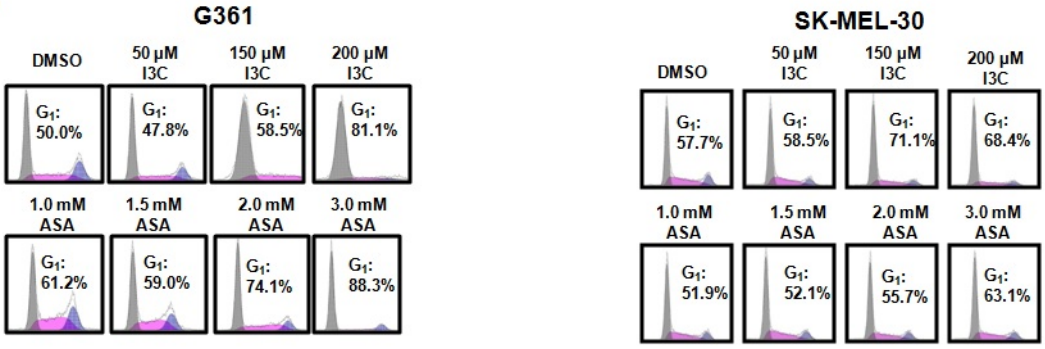
(A) The diagram describes the MITF-M interaction with its DNA binding site in the CDK2 promoter. CDK2 transcript expression in G361 cells treated with the indicated concentrations of aspirin (ASA) and I3C was determined by RT-PCR analysis in comparison to the GAPDH control transcript. Densitometry was utilized to measure relative intensity of the expressed CDK2 transcripts in each condition normalized to the ratio of CDK2:GAPDH in vehicle control treated cells. (B) Flow cytometry profiles of G361 cells (left panels) or SK-MEL-30 cells (right panels) treated with either aspirin (ASA) I3C for 48 hours. The average percent of the cell population in G_1 is indicated.

Fig 3A.

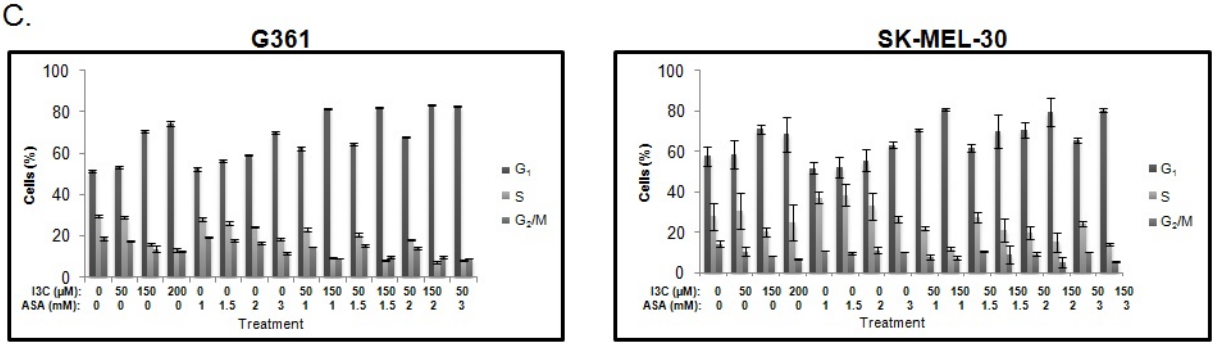

I3C
MITF-M
Aspirin
CDK2
CATGTG
-1315


ASA (mM): 0 0 0 0 1 1.5 2 3 1.0 1.5 2.0 3.0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
CDK2
GAPDH
Densitometry: 1 1.05 0.72 0.77 0.52 0.55 0.43 0.32 0.63 0.11 0.56 0.26 0.22 0.15 0.14 0.07

B.


G361
DMSO
50 μM I3C
150 μM I3C
200 μM I3C
G1: 50.0%
G1: 47.8%
G1: 58.5%
G1: 81.1%
1.0 mM ASA
1.5 mM ASA
2.0 mM ASA
3.0 mM ASA
G1: 61.2%
G1: 59.0%
G1: 74.1%
G1: 88.3%
SK-MEL-30
DMSO
50 μM I3C
150 μM I3C
200 μM I3C
G1: 57.7%
G1: 58.5%
G1: 71.1%
G1: 68.4%
1.0 mM ASA
1.5 mM ASA
2.0 mM ASA
3.0 mM ASA
G1: 51.9%
G1: 52.1%
G1: 55.7%
G1: 63.1%

C.


G361
SK-MEL-30
Cells (%)
100
80
60
40
20
0
G1
S
G2/M
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
ASA (mM): 0 0 0 0 1 1.5 2 3 1 1.5 1.5 2 2 3 3
Treatment

I3C and aspirin downregulate MITF-M transcript levels and promoter activity.

To assess the mechanism by which treatment with aspirin and/or I3C strongly down regulated MITF-M protein levels, the effects of these compounds on MITF-M transcript levels and promoter activity was examined in both G361 and SK-MEL-30 cells. As shown in Figure 4A, an RT-PCR analysis of cells treated for 48 hours with the indicated combinations of aspirin and I3C revealed a significant down regulation of MITF-M transcripts that strongly correlated with and likely accounts for the loss of MITF-M protein levels. In both cells lines, combinations of aspirin and I3C significantly attenuated transcript levels with the combination of 3.0 mM aspirin and 150 μM I3C almost completely ablating MITF-M transcript levels in the G361 cells, and significantly reducing MITF-M transcripts in SK-MEL-30 cells. Similar to the observed effects on cell proliferation and MITF-M protein levels, the downregulation of MITF-M transcripts was more pronounced in the G361 melanoma cells compared to the SK-MEL-30 melanoma cells. Also, for almost all of the sub-maximal combinations, the reduction of MITF-M transcripts was greater than the aggregate of the two respective treatments alone, indicating that the effects of the phytochemicals are able to induce a cooperative effect at the transcript level.

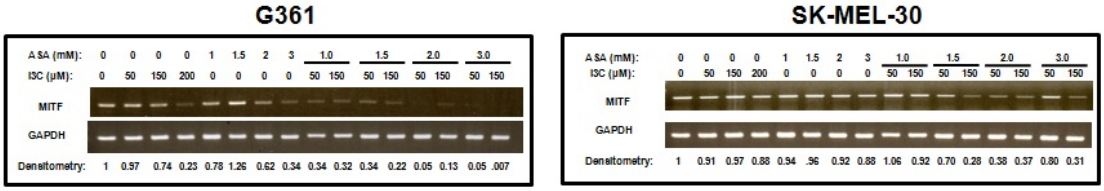
To determine if the aspirin and I3C downregulation of MITF-M transcript levels can be accounted for by decreased MITF-M promoter activity, both melanoma cell lines were transiently transfected with a MITF-M promoter-reporter plasmid containing the first 333 bp upstream of the RNA start site (pGL2-333/+120 MITF-M). This construct was previously demonstrated to be active in transfected melanoma cells (40), and contains multiple transcription factor binding sites, including a putative TCF/LEF1 site at position -199, and a BRN2 site at position -48 (see later section). Transfected melanoma cells were treated for 48 hours with the indicated combination of aspirin and I3C and reporter gene activity compared to vehicle control treated cells. As shown in Fig. 4B, treatment with either aspirin or I3C downregulated MITF-M promoter activity, demonstrating for the first time that aspirin and I3C signaling targets the MITF-M promoter. Furthermore, combinations of aspirin and I3C more significantly downregulate MITF-M promoter activity compared to the effects of either compound alone. This cooperative effect of combinations of aspirin and I3C suggest that each compound triggers distinct signaling pathways that target different regions of the MITF-M promoter, although relatively little is known about aspirin and I3C regulated signaling cascades that can potentially regulate MITF-M promoter activity.

Figure 4

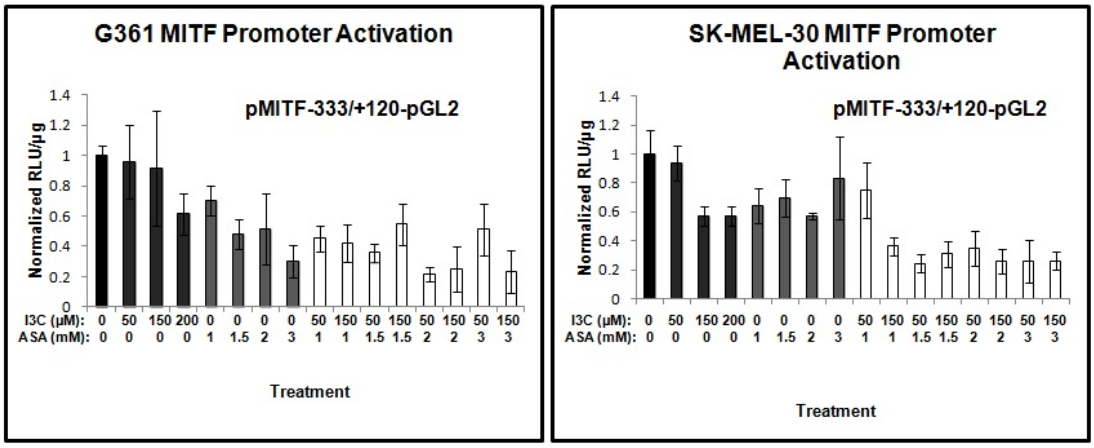
Combinational effects of aspirin and I3C on MITF-M transcript levels and promoter activity.

(A) G361 cells (left panel) and SK-MEL-30 cells (right panel) were treated with the concentrations of aspirin (ASA) and/or I3C for 48 hours and MITF-M transcript expression was determined by RT-PCR analysis in comparison to the GAPDH control transcript. Densitometry was utilized to measure relative intensity of the expressed MITF-M transcripts in each condition normalized to the ratio of MITF-M:GAPDH observed in the vehicle control. (B) G361 cells (left panel) and SK-MEL-30 cells (right panel) were transfected with the pGL2-333/+120 MITF-M reporter plasmid and then treated with the indicated concentrations of aspirin (ASA) or I3C for 48 hours. Luciferase specific activity was measured in the cell extracts and the bar graphs shows the results of three independent experiments in triplicate.

Fig. 4A.


G361
ASA (mM): 0 0 0 0 1 1.5 2 3 1.0 1.5 2.0 3.0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
MITF
GAPDH
Densitometry: 1 0.97 0.74 0.23 0.78 1.26 0.62 0.34 0.34 0.32 0.34 0.22 0.05 0.13 0.05 .007
SK-MEL-30
ASA (mM): 0 0 0 0 1 1.5 2 3 1.0 1.5 2.0 3.0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
MITF
GAPDH
Densitometry: 1 0.91 0.57 0.88 0.94 .96 0.92 0.88 1.06 0.92 0.70 0.28 0.38 0.37 0.80 0.31

B.


G361 MITF Promoter Activation
pMITF-333/+120-pGL2
Normalized RLU/μg
1.4
1.2
1
0.8
0.6
0.4
0.2
0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
ASA (mM): 0 0 0 0 0 1 1.5 2 3 1 1 1.5 1.5 2 2 3 3
Treatment
SK-MEL-30 MITF Promoter Activation
pMITF-333/+120-pGL2
Normalized RLU/μg
1.4
1.2
1
0.8
0.6
0.4
0.2
0
I3C (μM): 0 50 150 200 0 0 0 0 50 150 50 150 50 150 50 150
ASA (mM): 0 0 0 0 0 1 1.5 2 3 1 1 1.5 1.5 2 2 3 3
Treatment

Aspirin, but not I3C, disrupts production of members of the Wnt/β-catenin signaling pathway in melanoma cells

Aspirin has been shown to interfere with Wnt/β-catenin signaling in colorectal cancer cells by decreasing β-catenin protein stability and loss of beta-catenin regulated gene expression (41), suggesting the possibility that aspirin could be acting through this pathway in human melanoma cells. Because little is known about the effects of aspirin on Wnt/β-catenin signaling in melanoma cells, we examined the expression of several critical members of the Wnt/β-catenin signaling cascade in G361 melanoma cells treated for 48 hours with combinations of 3.0 mM aspirin and/or 200 μM I3C. Western blot analysis demonstrated that aspirin, but not I3C, strongly downregulated the protein levels of the LDL receptor-related protein 6 (LRP-6), a Wnt co-receptor, and of β-catenin (Fig. 5A). Furthermore, aspirin up regulated Axin levels, which has been shown to act as a negative regulator of β-catenin stability (42). Production of the transcription factor LEF1, which is a downstream effector of Wnt signaling and interacts with β-catenin to mediate its transcriptional activity, is unaffected by either aspirin or I3C treatment (data not shown).

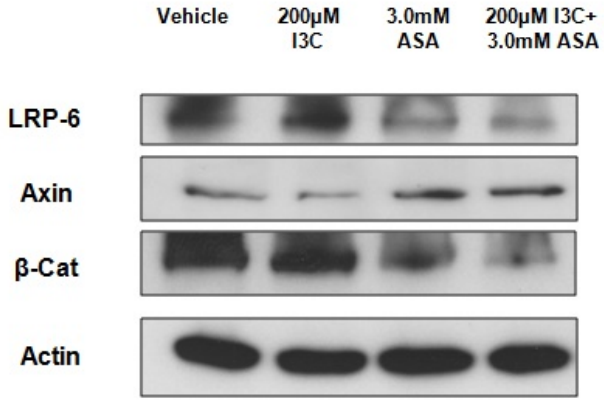
It is well established that aspirin inhibits the Cyclooxygenase 1 and 2 (COX-1/2) enzymes that are responsible for the production of prostaglandins (43). To confirm aspirin was indeed acting as expected in the melanoma cells, the levels of Prostaglandin E_2 (PGE_2) were determined in G361 melanoma cells treated for 48 hours with combinations of aspirin and/or I3C. As shown in Figure 5B, aspirin strongly downregulated PGE_2 levels in the presence or absence of I3C. Interestingly, when compared to the vehicle control treatment, I3C stimulated a small increase PGE_2 levels. Taken together, our results show that aspirin and I3C trigger distinct signaling pathways in human melanoma cells, and that the actions of these compounds do not interfere with each other.

Figure 5

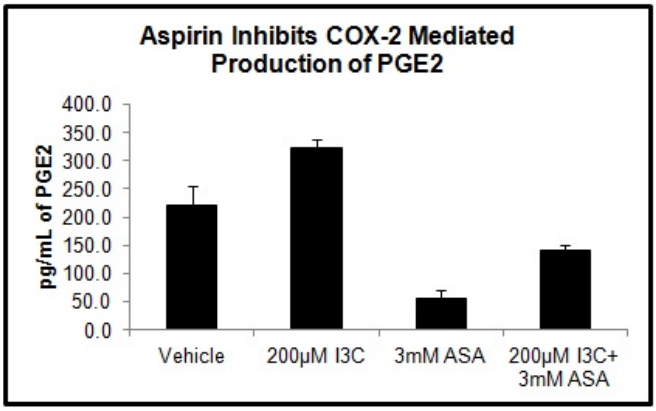
Differential regulation of Wnt signaling components and PGE2 levels by aspirin and/or I3C in melanoma cells.

G361 melanoma cells were treated with the indicated combinations of 200 μM I3C and/or 3.0 mM aspirin (ASA) or with the DMSO vehicle control for 48 hours. (A) Western blots of electrophoretically fractionated cell extracts were probed for components of the Wnt signaling pathway; LRP-6, Axin and β-catenin. (B) The production of PGE2 was determined as described in the methods and material section and averages of results from three independent experiments are shown in the bar graphs.

Fig. 5A.


Vehicle
200μM I3C
3.0mM ASA
200μM I3C+ 3.0mM ASA
LRP-6
Axin
β-Cat
Actin

B.


Aspirin Inhibits COX-2 Mediated Production of PGE2
pg/mL of PGE2
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Vehicle
200μM I3C
3mM ASA
200μM I3C+ 3mM ASA

Aspirin and I3C disrupt MITF-M promoter activity through distinct DNA binding sites downstream of either Wnt/β-catenin or MEK/ERK signaling pathways.

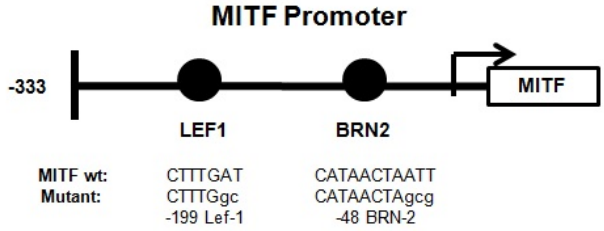
Conceivably, the aspirin disruption of Wnt/β-catenin signaling and the I3C disruption of BRAF/MEK/ERK signaling could account for the cooperative combinational effects of these compounds on MITF-M promoter activity. The -333 bp MITF promoter fragment within the pGL2-333/+120 MITF-M reporter plasmid contains a consensus TCF/LEF1 DNA binding site at position -199 bp (CTTTGAT), and a BRN2 binding site at position -48 bp (CATAACTAATT) of the MITF-M promoter. TCT/LEF1 is a downstream effectors of Wnt signaling in that upon Wnt activation, β-catenin translocates to the nucleus and binds to the TCF/LEF family of transcription factors forming a complex that recruits co-activators to Wnt regulated genes (44,45). The transcription factor BRN2 is downstream and activated by BRAF/MEK/ERK signaling (46), and we recently observed that I3C disrupts BRN2 nuclear localization and binding to the MITF-M promoter through the inhibition of oncogenic BRAF signaling (Kundu et al., submitted). We propose that the I3C downregulation of MEK/ERK signaling and the aspirin downregulation of Wnt/β-catenin signaling likely accounts for the cooperative downregulation of MITF-M promoter activity by aspirin and I3C. To test this possibility, combinations of the LEF1 and/or BRN2 canonical DNA binding sites in the MITF-M promoter were mutagenized in pMITF-333/+120-pGL2 at base pairs known to be critical for transcription factor binding (Fig. 6A) with one mutant construct containing only mutated LEF1 site (ΔLEF1) another construct was mutated only in the BRN2 site (ΔBRN2) and a third construct was mutated in both the LEF1 and BRN2 sites (ΔLEF1/ΔBRN2).

Each construct was linked to the luciferase reporter plasmid, transfected into either G361 or SK-MEL-30 melanoma cells and treated with combinations of 3.0 mM aspirin and/or 200 μM I3C for 48 hours. Reporter gene activity was measured and compared to vehicle control treated cells. As shown in Figures 6B and 6C, in both melanoma cell lines, combinations of aspirin and/or I3C treatment strongly downregulated MITF-M promoter activity. The cooperative effect of treatment with both compounds was particularly noticeable in the transfected G361 melanoma cells (Fig 6B, upper left panel). Mutation of the wild type LEF1 DNA binding site to CTTTGgc (forming the ΔLEF1 reporter plasmid) abrogated the aspirin downregulation of MITF-M promoter activity and the I3C downregulated response remained mostly intact in both cell lines (Figs 6B and 6C, lower left panels). In a complementary manner, mutation of the wild type BRN2 binding site to CATAACTAgcg (forming the ΔBRN2 reporter plasmid disrupted the I3C downregulation of MITF-M promoter activity and did not alter the aspirin downregulation of MITF-M promoter activity (Figs 6B and 6C, upper right panels). Finally, mutation of both the LEF1 and BRN2 canonical DNA binding sites (forming the ΔLEF/ΔBRN2 reporter plasmid) disrupted both the aspirin and I3C downregulation of MITF-M promoter activity (Figs 6B and 6C, lower right panels). Intriguingly, in G361 melanoma cells, treatment with aspirin, either alone or with I3C, had a mild stimulatory effect on MITF-M promoter activity that was observed only in the presence of the double mutated promoter. Taken together, our results demonstrate that the cooperative effects of aspirin and I3C on the downregulation of MITF-M promoter activity and gene expression can be accounted for by the selective targeting of distinct transcription factor binding sites in the MITF-M promoter.
Figure 6

LEF1 and BRN2 binding sites on MITF-M promoter required for combinatorial regulation of promoter activity by aspirin and I3C.

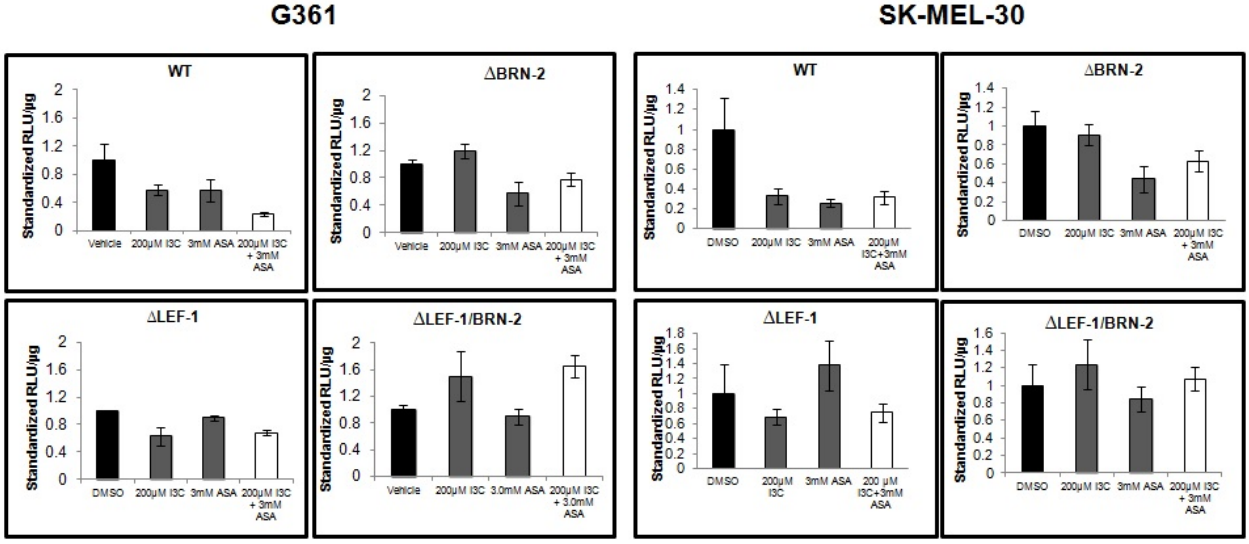
(A) Schematic diagram showing the sequences of the wild type LEF1 and BRN2 DNA binding sites in the MITF-M promoter and the sequence of the mutated LEF1 and BRN2 binding sites that were generated by PCR mutagenesis of the MITF-M promoter. (B) G361 cells or (C) SK-MEL-30 cells were transfected with the wild type pGL2-333/+120 MITF-M reporter plasmid, the ΔBRN2 pMITF-M-333/+120pGL2 reporter plasmid, the ΔLEF1 pMITF-M-333/+120pGL2 reporter plasmid or the ΔLEF1ΔBRN2 pMITF-M-333/+120pGL2 reporter plasmid containing mutations in both the LEF1 and BRN2 binding sites. The cells were then treated with the indicated combinations of 200 μM I3C and/or 3.0 mM aspirin (ASA) or with the DMSO vehicle control for 48 hours. Luciferase specific activity was measured in the cell extracts, standardized to vehicle control treated cells and the bar graphs show the average of results from three independent experiments in triplicate.

Fig. 6A.


MITF Promoter
-333
MITF
LEF1
BRN2
MITF wt: CTTTGAT CATAACTAATT
Mutant: CTTTGgc CATAACTAgcg
-199 Lef-1
-48 BRN-2

B.

C.


G361
SK-MEL-30
WT
ΔBRN-2
ΔLEF-1
ΔLEF-1/BRN-2
Standardized RLU/µg
Vehicle
200µM I3C
3mM ASA
200µM I3C + 3mM ASA
DMSO
3.0mM ASA
200µM I3C + 3.0mM ASA
200µM I3C+3mM ASA
200 µM I3C+3mM ASA

Discussion

MITF-M, the "master regulator" of melanocytes, displays a complex link to melanoma progression and proliferation (7,8), and is considered a lineage addiction oncogene (9) that is elevated in 10-20% primary melanoma tissue, and to an even higher degree in metastatic melanoma (47). Furthermore, MITF-M expression is negatively correlated with five-year survival of this cancer (9). A "rheostat model" has been proposed in which elevated expression levels of MITF-M lead to increased differentiation and cell cycle arrest, whereas, continued moderate levels of MITF-M expression signals melanoma cell survival and proliferation depending on the cellular mutation profile (7,36). It is also proposed that low to depleted levels of MITF-M expression is linked to quiescence or senescence, apoptosis and/or cell cycle arrest mediated by the loss of specific MITF-M target genes (48,49). Therefore, the management of MITF-M levels and transcriptional function is considered to be a critical factor in determining effectiveness and treatment outcomes of a given melanoma therapy (50). Attenuation or enhancement of MITF-M promoter activity represents an important cellular mechanism to acutely control MITF-M gene expression. Intriguingly, the MITF-M promoter includes canonical sequences for the binding of variety of transcription factors (40,51), which implicate several different cell signaling pathways that can potentially target MITF-M promoter activity and are potential targets of therapeutic strategies. However, relatively little is known about how potential anti-melanoma compounds influence the transcriptional control of MITF-M promoter activity.

Our studies have established that the cooperative anti-proliferative effects of aspirin and I3C in human melanoma cells trigger a significant downregulation of MITF-M gene expression and disruption of MITF-M promoter activity. The aspirin and I3C induced G_1 cell cycle arrest likely results from the loss of MITF-M expression because several essential G_1 acting cell cycle regulators that are downregulated in melanoma cells, such as CDK2 and CDK4, are MITF-M target genes (39,40). Aspirin and I3C targeted signaling pathways converge on the MITF-M promoter at distinct transcription factor binding sites in that the aspirin mediated portion of the response is dependent on the LEF1 binding site and the I3C effect is dependent on the BRN2 binding site. The LEF1/TCF family of transcription factors is a downstream effector of the canonical Wnt signaling pathway that forms a complex with nuclear localized β-catenin to promote MITF-M gene expression (52). Consistent with this concept, we have observed that aspirin disrupts Wnt signaling in melanoma cells by downregulating levels of the LRP-6 Wnt co-receptor protein and β-catenin as well as by elevating expression the Axin component of the destruction complex that can ubiquitin and target β-catenin for its proteasome mediated degradation. We propose that the aspirin-dependent the loss of β-catenin protein prevents its interaction with LEF1 on the MITF-M promoter.

The canonical Wnt/β-catenin signaling pathway in non-transformed cells is essential for cell processes such as maintaining cell polarity, movement, proliferation, differentiation, and survival. In many malignancies, an activating mutation in Wnt/β-catenin signaling occurs, which typically allows for inhibited activity of the destruction complex responsible for tagging cytoplasmic β-catenin for proteasomal destruction (53,54). The stabilized β-catenin translocates to the nucleus and binds with the TCF/LEF family of transcription factors, allowing for activation of downstream genes. Activating mutations are common in colorectal cancers as well as one third of melanomas, with that subset demonstrating significantly poorer prognosis than

those without aberrant Wnt signaling. In both types of cancer, activation of the Wnt/β-catenin pathway is an essential driver to cancer progression and chemoresistance (55,56). In metastatic melanoma cell lines, blocking β-catenin expression has been demonstrated to induce apoptosis, inhibit proliferation, and migration (57,58). In addition, it has recently been demonstrated that melanomas with active β-catenin signaling results in the absence of T-cell infiltration in the tumor microenvironment and inhibited host immune response, even blocking the activities of current immunotherapeutics (59).

Our results demonstrate for the first time that aspirin modulates the canonical Wnt signaling pathway in melanoma cells, although previous work has shown that aspirin interferes with Wnt/β-catenin signaling in colorectal cancer by increasing β-catenin phosphorylation at Ser33 and Ser37 decreased activity of protein phosphatase 2A (PP2A), providing a recognition site for the destruction complex and as a consequence, a decrease in β-catenin stability (60). The identification of aspirin as a cancer preventative agent emerged from the significant reductions in colorectal cancer and melanoma rates and mortality after a 5-year period in individuals who ingested daily doses of aspirin for cardiovascular problems (61). While low dose aspirin (81mg/day) is deemed relatively safe, higher doses (325mg/day or higher) such those used to treat sympotoms of inflamation and pain have the potential side effect of preventing clotting and increase the risk for gastric bleeding. As a result, strategies to decrease blood concentration of aspirin and its metabolites while maintaing its anti-cancer effects would be advantageous in a clinical setting. We have demonsrated that the *in vitro* concentrations of aspirin can be reduced to achive anti-proliferative effects in human melanoma cells by combining it with I3C, an anti-cancer phytochemical with few side-effects in humans (17). In contrast to aspirin, I3C required the BRN2 transcription factor site in the MITF-M promoter to mediate its portion of the combined effect on MITF-M gene expression and had relatively little effect on Wnt signaling or other aspirin responses, such as PGE2 production. We have recently observed that I3C disrupts downstream BRAF signaling resulting in the loss of BRN2 binding to and activating the MITF-M promoter (Kundu et al., Manuscript, submitted), which suggests that aspirin and I3C target the MITF-M promoter through distinct cell signaling cascades.

Combined cancer therapies have several advantages over the use of treatments with individual compounds. Treatments with combinations of anti-cancer drugs that act through different mechanisms can reduce the likelyhood of reisistant tumor cells escaping treatment and developing into a new resistant tumor. Furthermore, because of cooperative effects, a smaller dose of each drug may be given to achieve similar results, which can potentially reduce unintended side effects of higher drug concentrations. Given the high tolerance for I3C in humans, and the numerous clinical studies analyzing the tolerance of both low dose and high dose aspirin, the cooperative effects between I3C and aspirin demonstrated in our study provide evidence that this drug combination of realtively well tolerated phytochemicals has potential as an effective anti-melanoma adjunct therapy or perhaps as a preventative treatment. Also, combinations of aspirin and I3C display potent anti-proliferative effects in melanoma cells expressing either a wild type or oncogenic form of BRAF, suggesting that a range of melanomas with distinct mutation profiles will potentially be sensitive to combinations of aspirin and I3C as a therapeutic strategy.

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Chapter II

Antiproliferative Response Mediated by Artemisinin and its Semi-Synthetic Derivative Artesunate in Human Breast Cancer Cells

Abstract

Artemisinin, a naturally occurring sesquiterpene lactone, derived from the Artemisia annua plant, is a potent anti-malarial compound that has been demonstrated to have anticancer properties in multiple human cancer cell types, though the mechanisms in which it acts are still being explored. Its effects have been so encouraging that several semi-synthetic derivatives have been synthesized to better increase solubility and bioavailability. In this study, we demonstrate that Artemisinin (ART) and its semi-synthetic derivative Artesunate (AE) reduce the proliferation of T-47D human breast cancer cells, an early-stage, estrogen sensitive phenotype, through an induction of G_1 and G_2 cell cycle arrest, with AE able to induce significant arrest at much lower doses than the parental molecule. Concurrent with the cell cycle arrest, Artemisinin and Artesunate selectively downreugulated protein and transcript levels of Cyclin A, and its binding partners CDK1 and CDK2, while demonstrating no effect on other critical cell cycle regulators. Analysis of activation of Cyclin A promoter-luciferase constructs demonstrated that ablation of Cyclin A protein and transcripts was driven by an ART mediated reduction in promoter activity. Chromatin immunoprecipitation revealed that ART inhibited Sp1 binding to the Cyclin A promoter through ablation of Sp1 protein expression. Exogenous expression of Sp1 protein prevented the ART mediated effect on Cyclin A protein. Our results demonstrate that Artemisinin is able to exert control of the cell cycle via Sp1 mediated downregulation of Cyclin A, and possibly through attenuation of its CDK1 and CDK2 binding partners, and this represents a critical pathway in which ART controls human breast cancer growth.

Introduction

Breast cancer is the most common cancer found in women, and the second leading cause of cancer-related death (1). Generally, breast cancer can be classified as either hormone sensitive, which is marked by the presence of functional estrogen and/or progesterone receptor, and hormone insensitive which has at some point lost these (2,3). Two commonly used classes of chemotherapeutic treatments for hormone sensitive breast cancers are aromatase inhibitors, such as letrozole, and selective estrogen receptor modulators (SERMs), such as tamoxifen. While widely applied, these therapies are far from perfect because they can induce a variety of deleterious side effects including increased risk of blood clots, uterine cancer, and heart problems (4,5). Also, there and relatively high rates of recurrence upon treatment with these therapies, with approximately 40% of Estrogen Receptor positive patients eventually acquiring resistance (6,7). Many patients also develop a resistance to SERMs, through the mechanisms of resistance are not completely elucidated. In hormone insensitive cancers which include triple negative and HER2-positive, treatment options are more limited, though there has been some progress utilizing target-based therapy and the development of new immunoncology tools (8,9).

Naturally occurring plant compounds (phytochemicals) represent a class compounds that have yet to be fully explored for potential new therapeutics with relatively low levels of side effects and reduced rates of acquired resistance (10). One such family of phytochemicals is Artemisinin (ART) and its semi-synthetic derivatives. Artemisinin is found in the *Artimisia annua* plant, and has been used for hundreds of years to treat fever in China, but more recently has been one of the most widely used anti-malarial therapeutics on the market (11) with relatively low toxicity in humans (12). In addition to its anti-parasitic effects, Artemisinin and its derivatives have been shown to have anti-cancer effects in a variety of human cancers including colon, liver, breast, leukemia, melanoma, prostate, and ovarian (13-15). In rats exposed to the mammary carcinogen 7,12-dimethylbenz(α)anthracene, Artemisinin significantly inhibited the development of mammary cancers (16). Artesunate (AE) is one of the best studied derivatives of ART because of the addition of a hemisuccinate group, which allows for significantly increased water solubility and oral bioavailability, properties that potentially make it more potent (17). AE has been demonstrated to have cytotoxic activity in 55 cancer cell lines tested by the National Cancer Institute (18), has also been demonstrated to be more potent than its parental molecule in some systems (19), and is tolerated will in humans and animals.

Artemisinin and Artesunate contain an endoperoxide bridge that is cleaved by heme-iron, which forms free radicals. These free radicals then mediate multiple processes either in a parasitic or transformed cell such as; alkylation of heme molecules, inactivation of the sarcoplasmic/endoplasmic reticulum calcium pump, alkylation of cytosolic proteins, and disruption of mitochondrial functions (20,21). Even though many observed effects of Artemisinin derived compounds are due to this creation of free radicals, it has been demonstrated that they can act through other mechanisms such as inducing cell cycle arrest, apoptosis, and inhibition of angiogenesis (22-24). For example, the Firestone lab has demonstrated that Artemisinin can disrupt the ability of the transcription factor Sp1 to activate the CDK4 promoter in prostate cancer cells (25). This result and others like it demonstrate that Artemisinin and its derivatives exert proliferative control via the cell cycle on other cancer cell types. In this study, we report that Artemisinin, and its semi-synthetic derivative Artesunate induce a G_1 and G_2 cell cycle arrest in the luminal A ductal breast epithelial T-47D cell line via the downregulation of

cyclin A as well as both of its binding partners CDK1 and CDK2. This reduction in cyclin A expression is driven by a decrease in Cyclin A promoter activity driven by ART's ability to downregulate Sp1 protein levels, resulting in less activation of the promoter by that factor. We further demonstrate that exogenous expression of Sp1 confers resistance to the ART mediated reduction in Cyclin A, demonstrating a novel anticancer pathway driven by ART in human breast cancer cells.

Materials and Methods

Materials

Artemisinin, Artesunate, L-glutamine, penicillin, streptomycin, and dimethyl sulfoxide were purchased from Sigma Aldrich (St. Louis, MO). Iscove's Modified Dulbecco's Media was purchased from Lonza (Walkersville, MD). Fetal bovine serum was purchased from Gemini Bio-Products (Sacramento, CA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Bioscience (San Jose, CA). All other reagents are listed below and were of the highest purity available.

Cell Culture

T-47D human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Iscove's Modified Dulbecco's Media, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50U/mL streptomycin. Cells were incubated to subconfluency in a humidified chamber at 37^{o}C degrees containing 5% CO_2. Stock solutions of Artemisinin and Artesunate were dissolved in DMSO at 1000 times the desired concentration. The drugs were then diluted in media and then applied to the culture plates after washing the cells with phosphate-buffered saline (PBS) (Lonza).

Cell Proliferation Assay

Cells were plated onto 24-well tissue culture plates from Nunc (Denmark) at 70% confluency and treated as indicated in triplicate with DMSO vehicle, Artemisinin, or Artesunate, for 48 hours. Inhibition of proliferation was measured using the Dojindo Cell Counting Kit-8 (Rockville, Maryland) as per manufacturer's instructions. Briefly, 50µL of the CCK-8 solution was added to each well along with 450µL of full media and incubated for 2.5 hours. Absorbance was read at 450nm and percent inhibition was calculated by standardizing the average of each treatment triplicate to the average value of the vehicle control.

Western Blotting

After indicated treatments, western blots were performed as indicated previously (26). Rabbit anti-Cyclin A2 (sc-596), rabbit anti-CDK2 (sc-163), and mouse anti-Cdc2 (sc-54) were diluted 1:500 in Tris-Buffered Saline and Tween 20 (TBST). Mouse anti-Hsp90 from BD Transduction Laboratories (#610418 Franklin Lakes, NJ), rabbit anti-Actin from Cytoskeleton Inc. (#AAN01 Denver, CO) were diluted 1:1000 in TBST. Immunoreactive proteins were detected after a 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies diluted 3×10^{-4} in 1% nonfat dry milk dissolved in TBST. After 3 subsequent washes of 30 minutes each, blots were incubated with enhanced chemiluminescence reagents from Perkin Elmer Inc. (Waltham MA) for visualization on film.

Reverse Transcriptase PCR

Cells were treated with indicated doses and duration and then harvested in Trizol reagent from Sigma (St. Louis, MO) and RNA was extracted according to the manufacturer's protocol. 3.0 µg of total RNA was used for the reverse transcription reaction, utilizing Moloney Murine Leukemia Virus Reverse Transcriptase from Invitrogen (Carlsbad, CA) and random hexamers as per the manufacturer's protocol. 2µL of the cDNA was amplified in polymerase chain reaction using primers of the following sequences:

CDK2 Forward: 5'- CCA-GTA-CTG-CCA-TCC-GAG-AG -3'
CDK2 Reverse: 5'- CGG-CGA-GTC-ACC-ATC-TCA-GC -3'
CDK1 Forward: 5'-CTCCTGGTCAGTACATGGAT-3'
CDK1 Reverse: 5'-TGGAGTTGAGTAACGAGCTG-3',
Cyclin A Forward: 5'-TCC-ATG-TCA-GTG-CTG-AGA-GGC-3'
Cyclin A Reverse: 5'-GAA-GGT-CCS-TGA-GAC-AAG-GC-3'
GAPDH Forward: 5'-TCC-ACC-ACC-CTG-TTG-CTG-TC-3'
GAPDH Reverse: 5'-TGA-ACG-GGA-AGC-TCA-CTG-G-3'

PCR products were then run on a 1.1% agarose gels buffered with Tris borate-EDTA, and the products were then visualized with Ethidium Bromide from International Biotechnologies Inc. (New Haven CT).

Flow Cytometry

T-47D cells were plated in triplicate onto six-well culture dishes and grown to subconfluence. The cells were then treated with Artemisinin or Artesunate as indicated. The cells were then hypotonically lysed in 300 µL of DNA staining solution (0.5mg/mL propidium iodide, 0.1% sodium citrate, and .05% Triton X-100. Nuclear fluorescence (585nm) was measured on a Beckman-Coulter EPICS XL instrument with laser output adjusted to deliver 15 megawatts at 488nm. For each sample 10,000 nuclei were analyzed and the percentage of cells in G_1, S, G_2/M phase of the cell cycle was determined by analysis with Multicycle provided by Phoenix Flow Systems in the Cancer Research Laboratory, Flow Cytometry Facility of the University of California, Berkeley.

Luciferase Assays

All of the Cyclin A promoter constructs were a kind gift from Dr. Michael Birrer at MGH Cancer Center. T-47D cells were grown to 60% confluence in six-well cell culture plates and then transfected using Fugene 6 from Roche (Pleasanton, CA) as per the manufacturer's instructions. Twenty-four hours after the transfection, cells were then treated with either DMSO as a vehicle control or 300 µM Artemisinin for a period of 48 hours. Cells were then lysed and luciferase activity was evaluated using the Promega luciferase assay kit (Promega) and a luminometer. Relative luciferase activities were then normalized to the protein input with standard error. This procedure was performed three separate times with triplicate samples of each treatment to verify reproducibility.
Chromatin Immuno-Precipitation (ChIP)

T-47D cells were grown to subconfluence and treated for 48 hours with 300 μM ART or DMSO. DNA was crosslinked to proteins through the addition of 1% formaldehyde and incubated at room temperature for 5 minutes. The fixation reaction was quenched with addition of glycine for a final concentration of 125 mM and incubated for 5 minutes at room temperature. Cells were then harvested and lysed with chromatin immunoprecipitation lysis buffer (50 mM NaCl, 1% Triton X-100 and 0.1% sodium deoxycholate) with protease inhibitors (50g/mL phenylmethylsulfonyl fluoride, 10g/mL, aprotinin, 5g/mL leupeptin, 0.1g/mL NaF, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 0.1 mM glycerol phosphate). Cells were sonicated and supernatants were standardized using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). 1mg protein was used for each IP. Protein-DNA complexes were incubated overnight with 15μL antibody recognizing Ets-1 from Santa Cruz Biotechnology. Complexes were precipitated by adding Sepharose-G beads from GE Healthcare (Piscataway, NJ) and incubating at 4^{o} for 1 hour. Beads were then washed 2x with CHIP lysis buffer, followed by 2x with CHIP wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate and 1 mM ethylendiaminetetraacetic acid). Samples were eluted with CHIP elution buffer (50 mM Tris pH 8.0, 1% sodium dodecyl sulfate, and 10 mM ethylenediaminetetraacetic acid) at 65^{o} for 18 hours. PCR was carried out in a total of 50μL using the following primers, Forward: 5'- CCT-AAA-TCC-TAC-CTC-TCC-CC-3' Reverse: 5'-CCC-GCG-ACT-ATT-GAA-ATG-GAC-C-3'. Products were visualized on a 1.2% agarose gel buffered with Tris borate-EDTA, and the products were then visualized with Ethidium Bromide.

Results

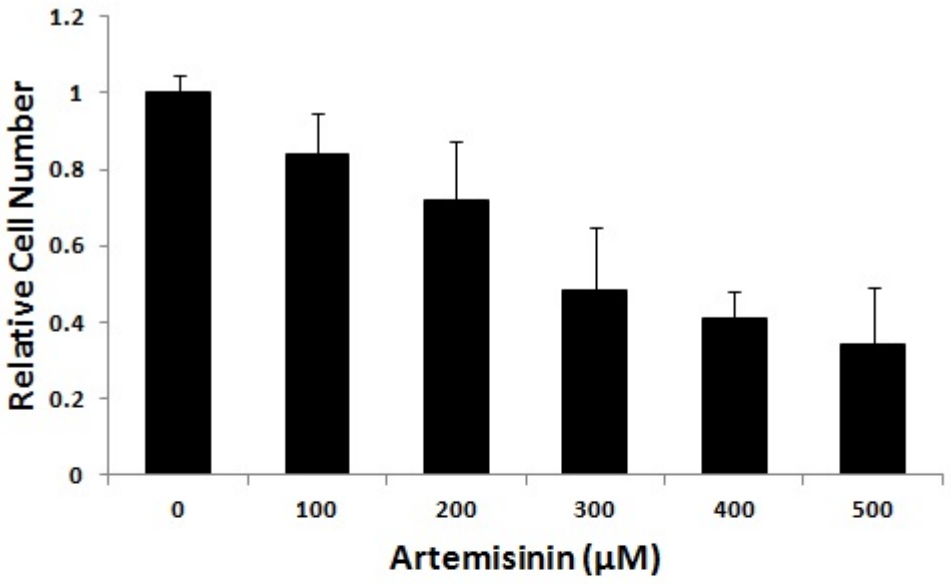
Artemisinin and Artesunate inhibit cell proliferation in T-47D human breast cancer cells

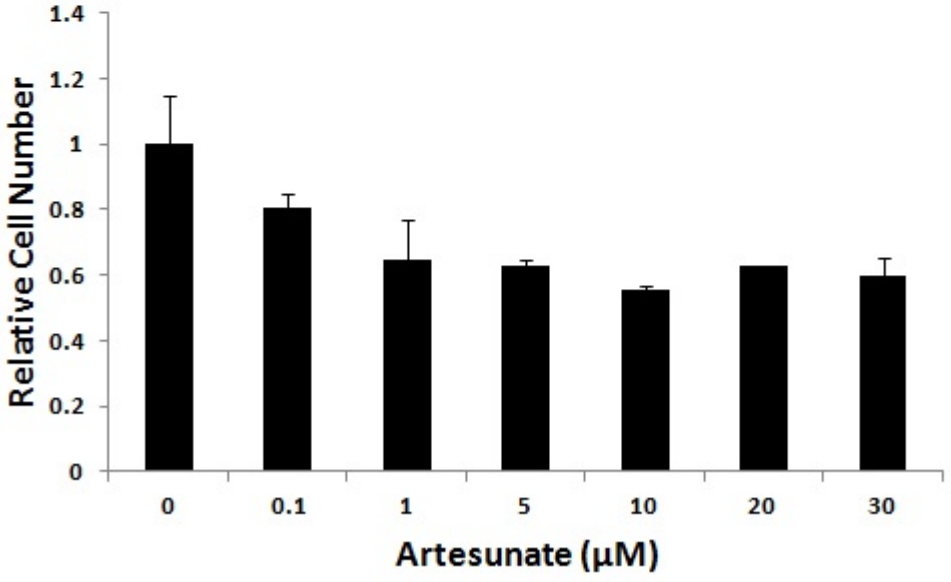
The antiproliferative effects of ART and AE on the T-47D luminal A type human breast cancer cell line (27) was initially assessed by treating cells with increasing concentrations of both compounds (up to 500 μM for ART and 30 μM for AE) or the vehicle control and quantifying relative cell number after 48 hour of treatment (Figure 1). This experiment was also performed at 6 hour, 24, and 72 hour time points, the Artemisinin-derived effect was noted at 24 hours, and was no greater at 72 hours than 48 hours (data not shown). In both cases, ART and AE were able to decrease overall cell number when compared to the vehicle control, with relative cell number reaching about half of the vehicle control when treated with 300 μM ART (top panel) and 10 μM AE (bottom panel), demonstrating that AE is much more potent in regulating cell proliferation than ART. These concentrations are consistent with those seen in the literature to induce growth arrest but not a significant amount of apoptosis.

Figure 1

Artemisinin and Artesunate reduce T-47D cell number.

ART and AE both reduce proliferation of T-47D human breast cancer cells *in vitro*. Equal numbers (approx. 300,000 cells/well) of cells were seeded and treated with indicated doses for 48 hours, and the final relative cell number was determined via addition of CCK-8 solution (a water soluble tetrazolium salt), subsequent incubation for 3 hours at $37^{o}C$ and 5.0% CO_2, and measured absorbance at 450nm. Absorbance values were normalized to the vehicle control, and each condition was measured in triplicate.


Relative Cell Number
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
Artemisinin (μM)


Relative Cell Number
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.1
1
5
10
20
30
Artesunate (μM)

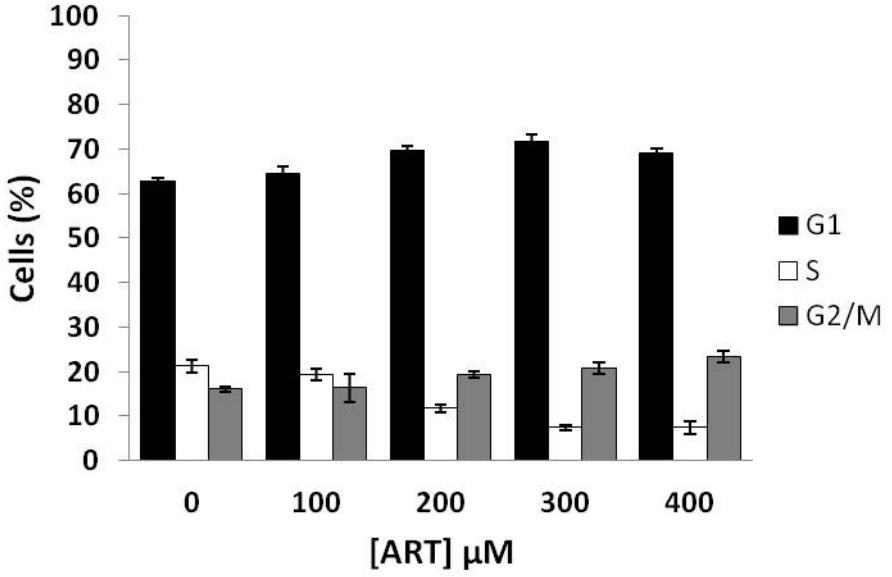
ART and AE both induce G_1 and G_2 cell cycle arrest in T-47D cells

The inhibition of proliferation by ART and AE could derive from several mechanisms including induction of apoptosis, induction of cell cycle arrest, or senescence. To test whether these compounds can affect the cell cycle in this cell line, T-47D cells were treated with increasing doses of ART or AE for 48 hours, and then harvested. Cellular DNA was stained with propidium iodide and DNA content was measured to determine cell cycle distribution of each treatment (Figure 2). Artemisinin was able to induce a 10% increase in cells in the G_1 phase at a concentration of 300 μM (top panel). In addition, the treatment caused a small but significant increase in cells in the G_2/M phase, which is unusual for compounds that induce cell cycle arrest. When compared to vehicle control, treatment with 5 μM AE (bottom panel) induced a 17% increase in cells in the G_1 phase, and again a small but significant increase in cells in the G_2/M phase was observed. In both conditions there was not a significant increase in sub-G_1 DNA fragments, an indicator of apoptosis. From this, we conclude that the decrease in cell number was due mainly to an induction of cell cycle arrest rather than apoptosis. Using the doses of 300 μM ART and 5 μM AE, which were most effective while causing a negligible amount of cell death, cells were treated at time points ranging from 24-72 hours (Figure 3). For both compounds, the largest change in arrested cells was observed at 48 hours, with 72 hour treatment yielding very similar results.

Figure 2

Artemisinin and Artesunate induce a G_1 and G_2 cell cycle arrest in T-47D cells in a dose dependent manner.

T-47D cells were treated with indicated concentrations of ART (top panel) and AE (bottom) for 48 hours, and the DNA was stained with propidium iodide and measured by flow cytometry. The bar graphs represent the percent of the total population in each phase of the cell cycle, and each condition was performed in triplicate in three independent experiments.


100
90
80
70
60
50
40
30
20
10
0
Cells (%)
0
100
200
300
400
[ART] µM
G1
S
G2/M

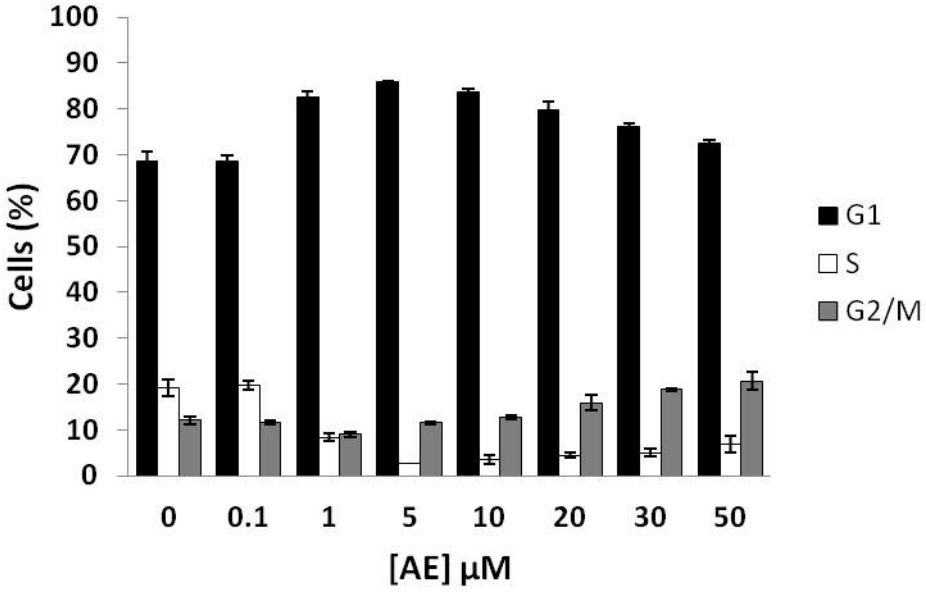
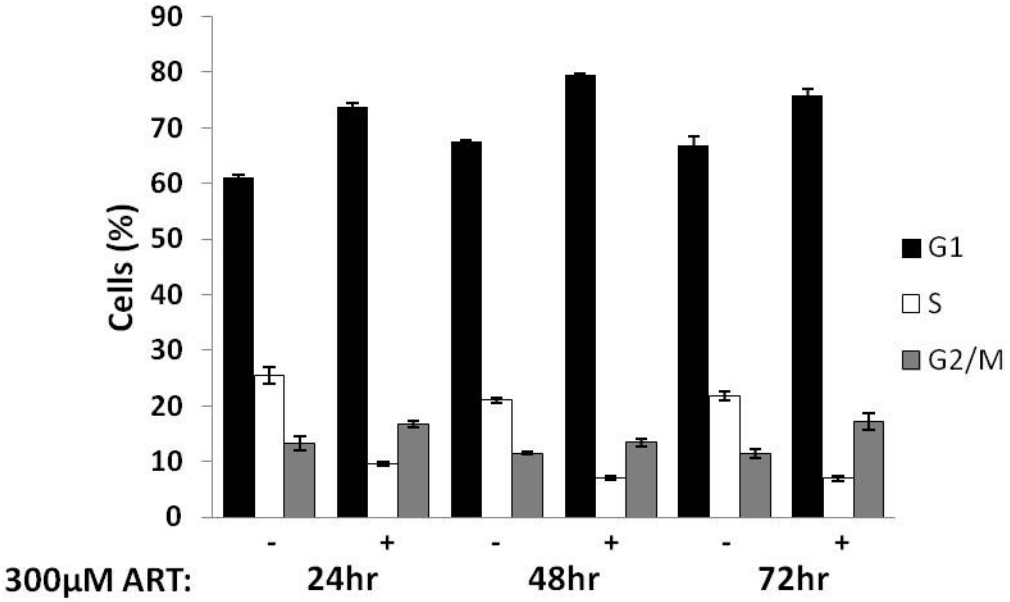
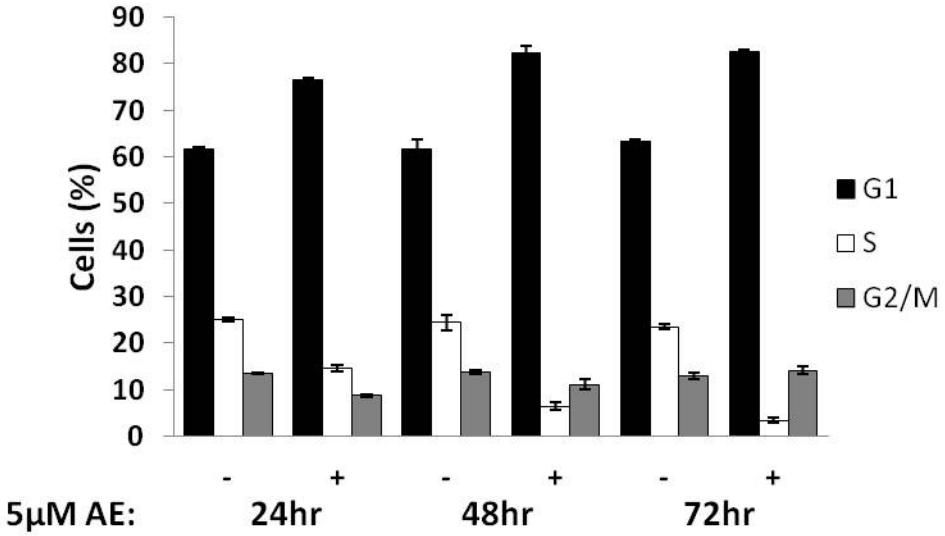

100
90
80
70
60
50
40
30
20
10
0
Cells (%)
0
0.1
1
5
10
20
30
50
[AE] µM
G1
S
G2/M

Figure 3

ART and AE induce maximum cell cycle arrest by 48 hours in culture.

T-47D cells were treated with either 300 μM ART (top panel) or 5 μM AE for either 24, 48, or 72 hours, after which the cellular DNA was stained with propidium iodide and measured by flow cytometry. The bars represent the percent of the overall population in each phase of the cell cycle, and the experiment was performed in triplicate in three independent experiments.


90
80
70
60
50
40
30
20
10
0
Cells (%)
G1
S
G2/M
-
+
-
+
-
+
300μM ART:
24hr
48hr
72hr


90
80
70
60
50
40
30
20
10
0
Cells (%)
G1
S
G2/M
-
+
-
+
-
+
5μM AE:
24hr
48hr
72hr

Cyclin A, CDK1, CDK2 protein and mRNA levels are downregulated in a dose and time dependent manner upon T-47D treatment with ART and AE

To further characterize the ART and AE derived cell cycle arrest, T-47D cells were treated with increasing doses of ART and AE, total cell lysates were then isolated and subjected to immunoblotting with probes for several key cell cycle factors (Figure 4). For both ART and AE, expression levels of Cyclin A, and its binding partners CDK1 and CDK2 were downregulated in a dose dependent manner. 300 μM ART induced the maximum downregulation of those proteins, while 5 μM AE was necessary for maximum effect. This again demonstrates that AE is far more potent than its parental molecule. A time course treatment was also performed analyzing the expression of Cyclin A, CDK1, and CDK2 after 24, 48, and 72 hours to determine the optimal length of treatment (Figure 5). For both ART and AE, 48 hours was sufficient to induce maximum downregulation of protein expression.

Control of the cell cycle, especially of the Restriction point and G_2/M transition is conferred by many factors in the cell, and to be sure that the observed arrest in these cells was due to attenuation of Cyclin A, CDK1 and CDK2 expression, the expression of other critical cell cycle factors' was analyzed. Upon treatment with the same dose response of ART as in figure 4, the expression of G_1 cyclins and CDKs Cyclin E (both high and low molecular weight), Cyclin D1, CDK6 was analyzed and found not to be affected (Figure 6). CyclinD_1 binds to and controls the kinase of activity of CDK6 in the G1 phase, and allows it to phosphorylate retinoblastoma (Rb). Towards the end of the G1 phase, Cyclin D is replaced by Cyclin E, and the Cyclin E/CDK2 complex hyperphosphoylates Rb, which allows for the release of the E2F family of transcription factors which regulate genes that allow for continuation of the cell cycle. Cyclin E is also necessary stabilize critical factors that form the pre replication machinery necessary for DNA replication in the S phase (28). Cdc25A is a phosphatase that is able to remove an inhibitory phosphate group at Thr-14 on CDK2 activating it (29). In addition, expression of Cyclin B_1, a critical regulator of G_2 phase, was not affected by ART treatment. Accordingly, these data suggest induction of G_1 arrest could be induced by the loss of either Cyclin A or CDK2 alone, or possibly the two in combination, though it is possible that the effect could be conferred by attenuation of kinase or phosphatase activity of the CDKs and Cdc25A respectively. Furthermore, the slight increase in G_2/M arrested cells might also be due to a loss in Cyclin A and its S/G_2 phase mediator, CDK1, and not a decrease in CyclinB_1 expression.

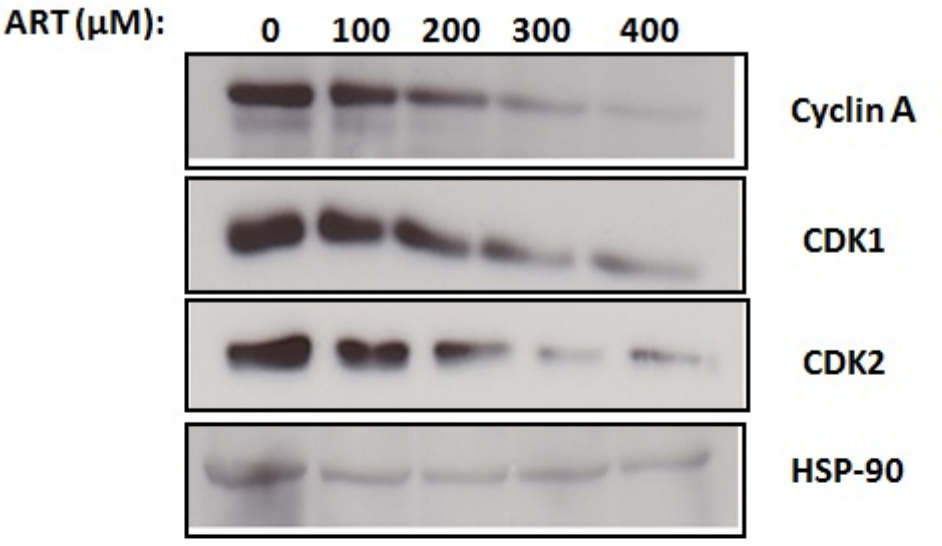
RT-PCR analysis of total RNA isolated from cells that were either untreated or treated with ART or AE revealed that the transcripts of Cyclin A, CDK1, and CDK2 were all downregulated in a dose dependent manner (Figure 7). Once again, 300 μM produced the maximum downregulation for all transcript levels (top panel). Interestingly, only 1.0 μM AE was necessary to completely ablate Cyclin A mRNA, while 5 μM was necessary to produce the maximum downregulation of the other cell cycle factors (bottom panel). RT-PCR was also performed on cells treated for 24, 48, and 72 hours to see if the kinetics of mRNA downregulation coincided with the downregulation of protein levels (Figure 8). Indeed, a significant attenuation of transcripts was noted at the 48 hour time point, similar to what was observed at that time point for protein expression, but interestingly, cells treated for 72 hours has less attenuated transcript levels modestly more than the 48 hour time point, which is not reflected in the protein expression levels. It is possible that ART and AE have a minor effect on protein stability in addition to the

attenuation of mRNA transcripts, thus allowing for full downregulation of protein expression at an earlier time point than observed by rt-PCR, but from this data we can conclude that most of the effect on protein expression was driven by a decrease in transcript levels.

Figure 4

ART and AE regulate Cyclin A, CDK1, CDK2 protein

T-47D cells were treated at indicated doses of ART (top panel) or AE (bottom panel) for 48 hours. Total cell extracts were isolated and electrophoretically fractionated and probed for the indicated cell cycle genes, HSP-90 protein levels were used as a gel loading control.


ART (μM):
0
100
200
300
400
Cyclin A
CDK1
CDK2
HSP-90

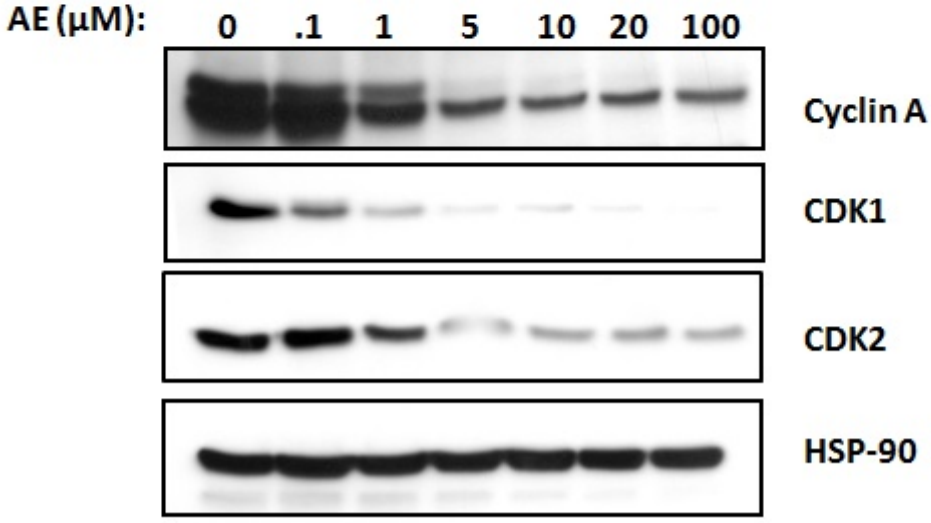

AE (μM):
0
.1
1
5
10
20
100
Cyclin A
CDK1
CDK2
HSP-90

Figure 5

ART and AE downregulate Cyclin A, CDK1, CDK2 protein expression in a time dependent manner, with maximum downregulation at 72 hours

T-47D cells were treated with either 300 μM ART (top panel) or 5 μM AE (bottom panel) for 24, 48, or 72 hours. Western blotting was performed on the cell extracts and probed for cyclin A, CDK1, or CDK2. HSP-90 was used as a loading control.

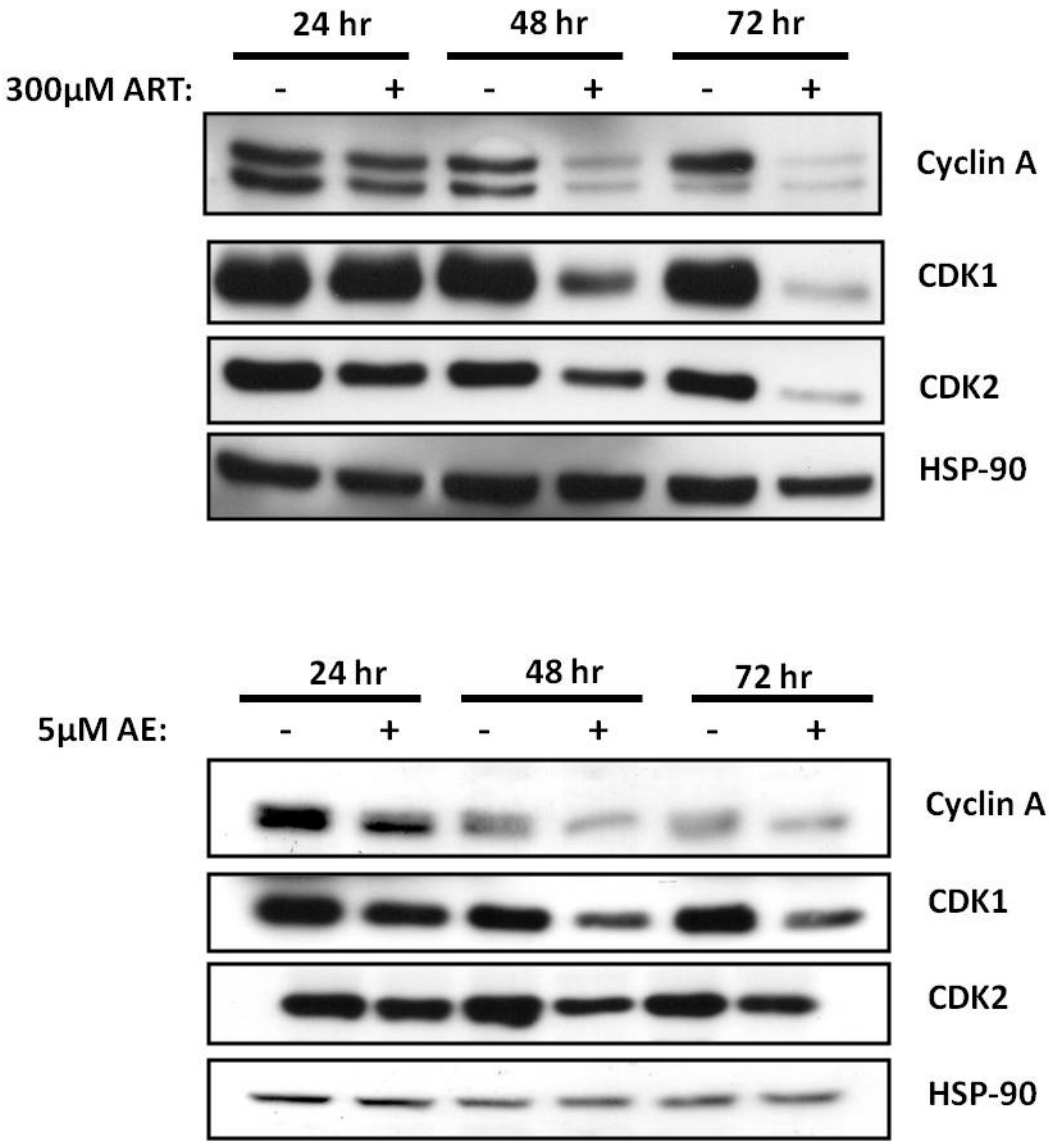

24 hr
48 hr
72 hr
300μM ART:
- + - + - +
Cyclin A
CDK1
CDK2
HSP-90
24 hr
48 hr
72 hr
5μM AE:
- + - + - +
Cyclin A
CDK1
CDK2
HSP-90

Figure 6

ART does not effect the expression of other critical G_1/S regulators

T-47D cells were treated with either the vehicle control or increasing doses of ART. Protein expression of $CyclinB_{1,}$ CyclinD1, CDK6, Cdc25A, and high and low molecular weight forms of Cyclin E were analyzed. Actin was used as a loading control.

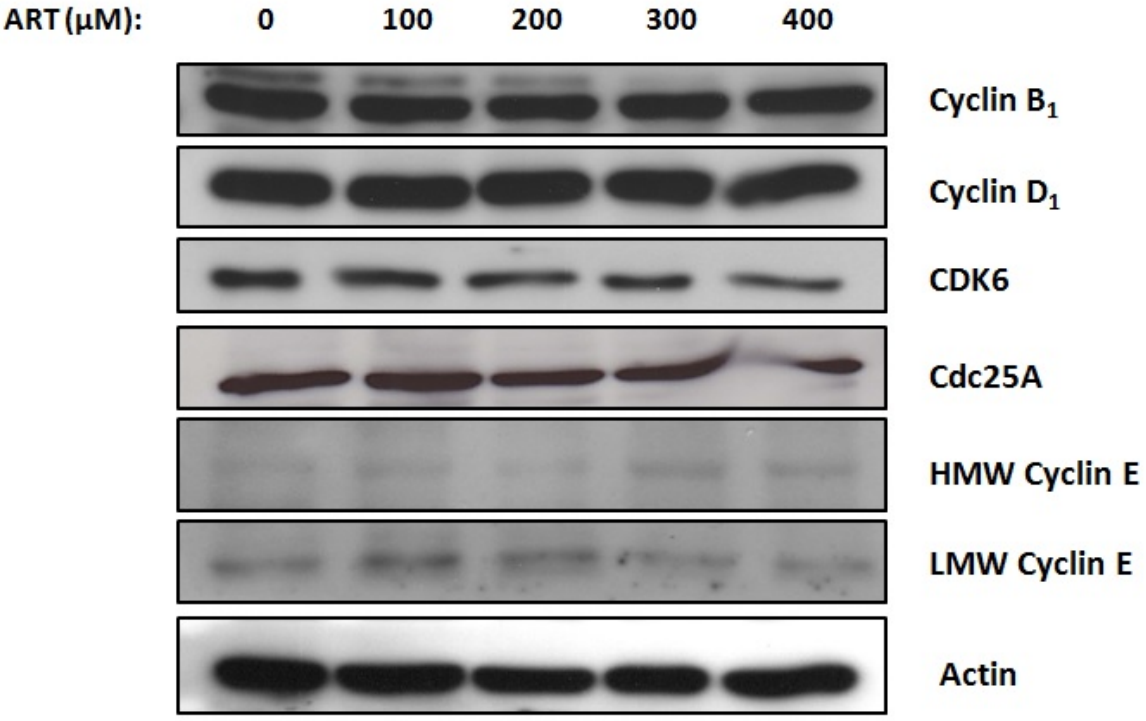
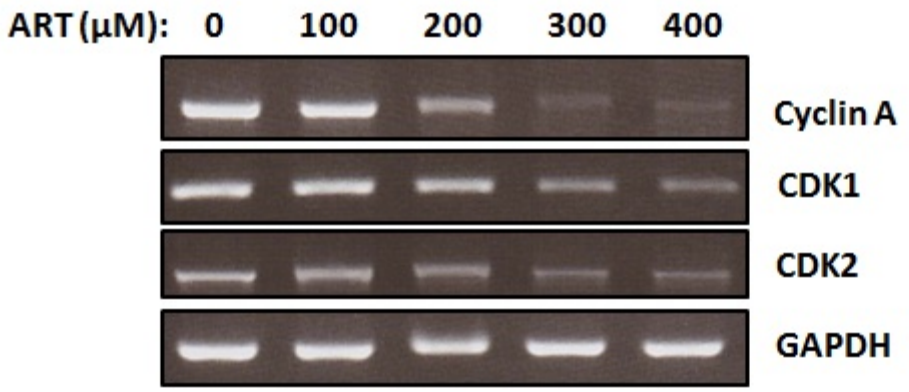

ART (μM):
0
100
200
300
400
Cyclin B1
Cyclin D1
CDK6
Cdc25A
HMW Cyclin E
LMW Cyclin E
Actin

Figure 7

ART and AE downregulate cyclin A, CDK1, CDK2 mRNA in a dose dependent manner

T-47D cells were treated at indicated doses of ART or AE for 48 hours and total RNA was isolated. Transcript levels of cyclin A, CDK1, and CDK2 were determined by reverse transcription-polymerase chain reaction using specific primers for each target. The products were loaded onto a 1.1% agarose gel and fractionated by electrophoresis, and then visualized on an ultraviolet transilluminator. GAPDH was used as a gel loading control.


ART (μM): 0 100 200 300 400
Cyclin A
CDK1
CDK2
GAPDH

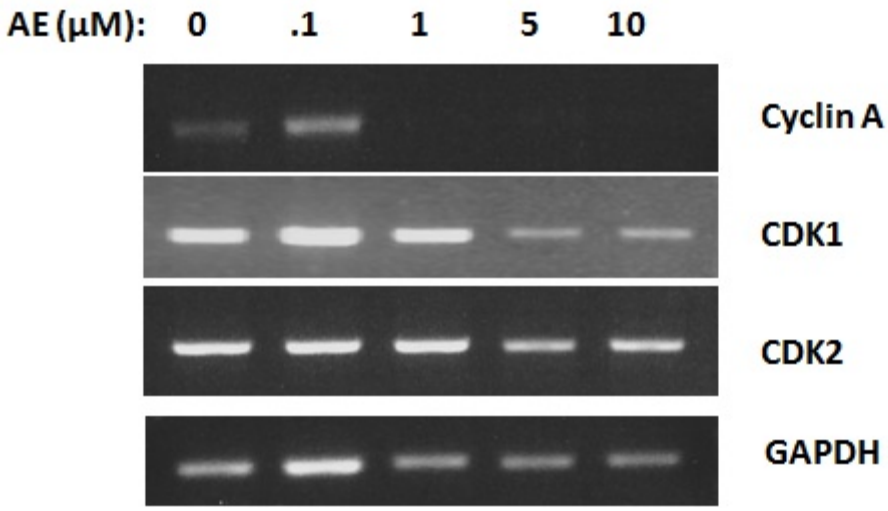
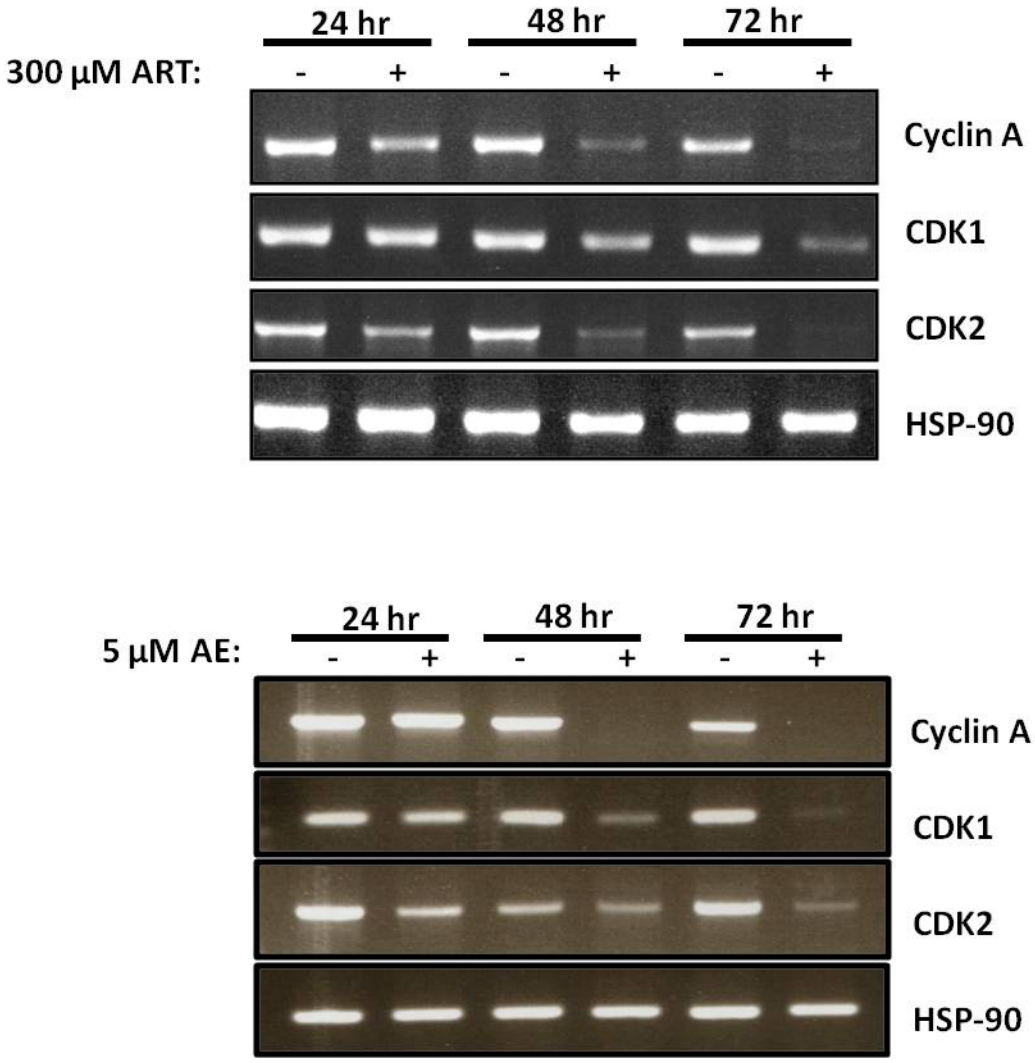

AE (μM): 0 .1 1 5 10
Cyclin A
CDK1
CDK2
GAPDH

Figure 8

ART and AE downregulate cyclin A, CDK1, CDK2 transcript levels in a time dependent manner, with maximum downregulation at 72 hours

T-47D cells were treated with either 300 μM ART (top panel) or 5 μM AE for either 24, 48, or 72 hours. Transcript levels of cyclin A, CDK1, and CDK2 were determined by reverse transcription-polymerase chain reaction using specific primers for each target. The products were loaded onto a 1.1% agarose gel and fractionated by electrophoresis, and then visualized on an ultraviolet transilluminator. GAPDH was used as a gel loading control.


24 hr
48 hr
72 hr
300 µM ART:
- + - + - +
Cyclin A
CDK1
CDK2
HSP-90
24 hr
48 hr
72 hr
5 µM AE:
- + - + - +
Cyclin A
CDK1
CDK2
HSP-90

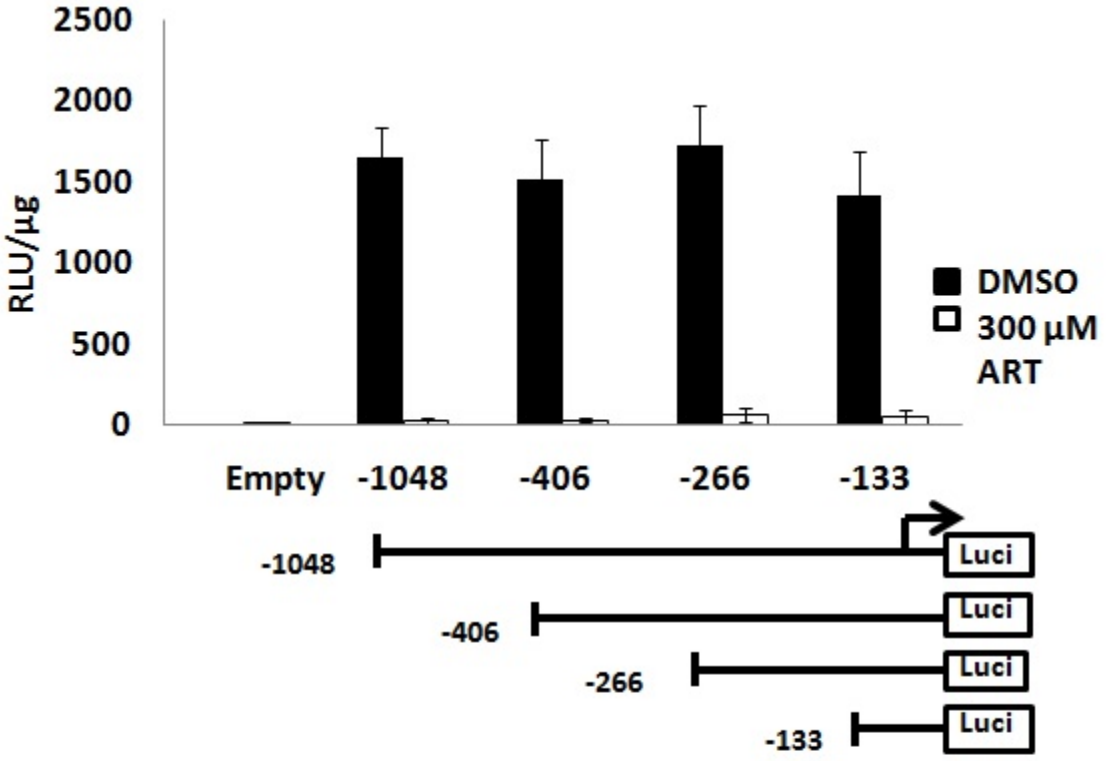
ART attenuates Cyclin A proximal promoter activity

Because of the importance of Cyclin A in controlling the G_1/S transition as well as the S/G_2 transition, and its almost complete downregulation of Cyclin A transcript levels by ART and AE, we further analyzed the effects of ART on the activity of the Cyclin A promoter. To do this, and to identify the ART-responsive region of the promoter, we transfected T-47D cells with serial truncated portions of the Cyclin A promoter region, fused to a luciferase reporter gene in a pGL2 plasmid backbone. A total of 4 promoter constructs were utilized; -1048/+205, -406/+205, -266/+205, and -133/+205 bp away from the transcriptional start site. After transfection, cells were treated for 48 hours with either vehicle control or 300 μM ART, and luciferase activity was measured from the lysed cell extracts. The activity of each promoter construct reduced almost to background levels when the cells were treated with ART versus the vehicle control (Figure 9). Because the smallest portion of the promoter was still regulated by ART, the ART responsive region must be contained within that region -133/+205 from the transcriptional start site.

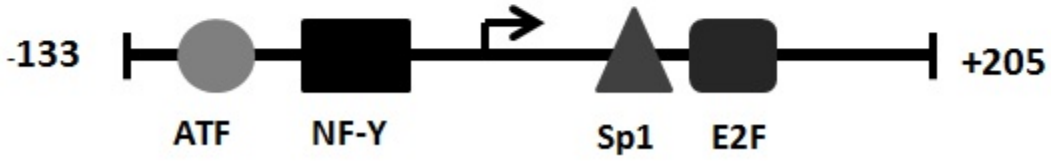
Figure 9

Artemisinin regulates Cyclin A transcriptional activity

T-47D cells were transfected with serial truncations of the Cyclin A promoter linked to a Luciferase gene in a pGL2 –Basic Vector (pGL2-***-CycA-LUC) or pGL2-Basic (negative control). After a 24 hour incubation, cells were treated with either the vehicle control or 300 μM ART for 48 hours. Cells were then lysed, and lysates were assayed for luciferase activity (top panel). Activity was normalized to protein input.



Cyclin A Promoter Constructs



ART mediated control of Cyclin A promoter activation mediated by a decrease in Sp1 protein expression

There are several notable transcription factor binding sites within the ART mediated range of the 5' region of the Cyclin A gene including ATF (30), NF-Y (31), Sp1 (31), and E2F (32) (Figure 9, bottom panel). While all of those factors could be responsible for the ART mediated effect on Cyclin A promoter activity, the Sp1 site was of particular interest since the Firestone lab has demonstrated that Artemisinin can control Sp1 binding of target gene promoters in prostate cancer cells (25). To see if something similar might be happening in T-47D cells we first needed to determine if treatment with Artemisinin could attenuate Sp1 protein expression. We treated T-47D cells with increasing dose of ART for 48 hours and immunoblotted for Sp1 expression. Interestingly, Sp1 levels were attenuated in a dose dependent manner in T-47D cells, an effect not previously observed in other cell lines (Figure 10, top panel). Chromatin immunoprecipitation was used to determine whether the ART mediated downregulation of Sp1 protein affects the endogenous Sp1 interactions with the 5' upstream region of the Cyclin A gene. As shown in Figure 10 (middle panel) in 48 hour ART treated cells, binding of Sp1 to the Cyclin A proximal promoter was significantly disrupted compared to untreated cells. Thus, ART mediated control of Sp1 protein expression is most likely the cause of decreased Sp1 binding to the Cyclin A promoter, which potentially accounts for the regulation of Cyclin A promoter activation. It should be noted that these data is preliminary, and further repetitions should be completed.

If the disruption in Sp1 binding to the proximal Cyclin A promoter is what is responsible for disruption in promoter activation and overall expression, a key prediction is that overexpression of exogenous Sp1 should rescue ART mediated downregulation of Cyclin A protein expression. T-47D cells were transiently transfected with either CMV-neo empty vector or CMV-Sp1 expression vector. After a 24 hour incubation, the cells were then treated with either the vehicle control or 300 μM ART for 48 hours, and then the cells were lysed and lysates were examined for Sp1 and Cyclin A protein expression via western blot (Figure 10, bottom panel). In both the cells transfected with CMV-neo and CMV-Sp1, ART decreased Sp1 expression when compared to the internal control, however in the cells transfected with the CMV-Sp1 there was still enough exogenous Sp1 to be comparable to the endogenous levels in the control empty vector cells treated with the vehicle control. Cyclin A expression was significantly reduced by ART when compared to the vehicle control in the cells transfected with the empty vector. Contrastingly, Cyclin A levels were almost completely unaffected by ART when compared to the vehicle control in the cells transfected with CMV-Sp1, demonstrating that the ART-mediated downregulation of Cyclin A expression was effectively blocked by Sp1 overexpression.

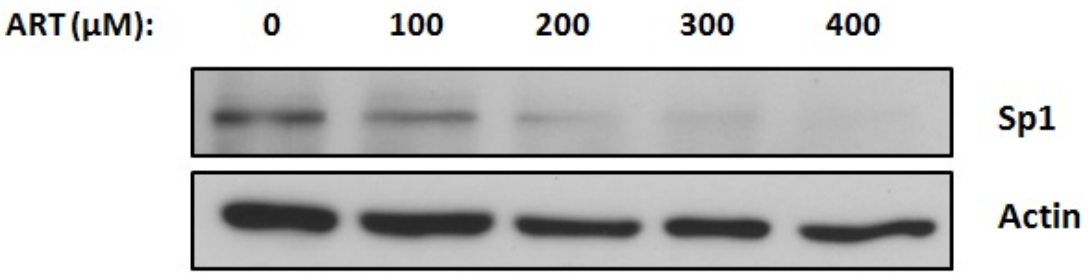
Figure 10

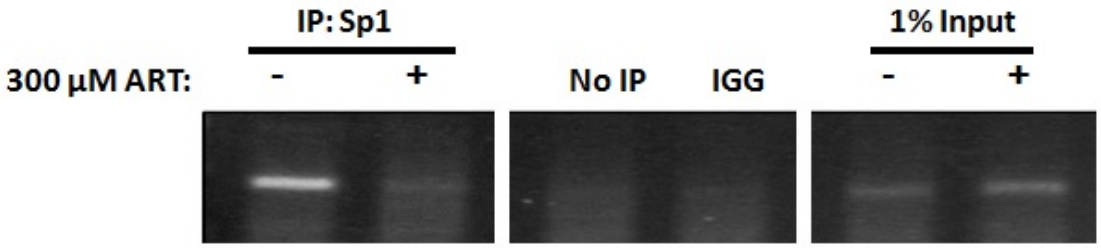
Sp1 binding mediates ART effect on Cyclin A promoter activation

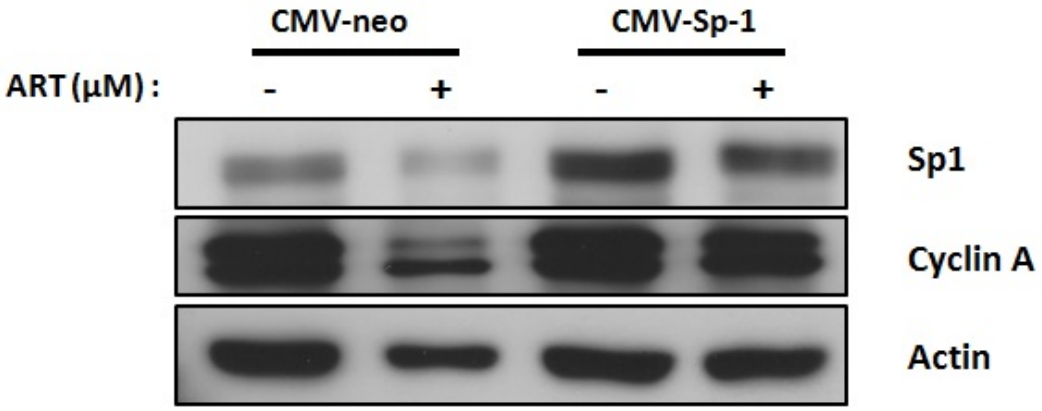
Top panel: Cells were treated with increasing doses of ART, and Sp1 levels were down regulated in a dose dependent manner.

Middle panel: Chromatin was isolated from T-47D cells treated with or without 300 μM ART for 48 hours. Sp1 was immunoprecipitated from total cell extracts using Sepharose G bound to anti-Sp1 antibody, and DNA eluted from Ep1 was amplified using indicated oligonucleotide primers. The IGG control utilized a anti-GFP antibody instead of Sp1. Input samples represent total genomic DNA from each treatment (loading control).

Bottom panel: T-47D cells were transfected with CMV-Sp1 or the CMV-neo vector and treated with or without 300 μM ART for 48 hours. Total cell lysates were electrophoretically fractionated and analyzed by Western blot for levels of Sp1, Cyclin A, and Actin (loading control).


ART (μM):
0
100
200
300
400
Sp1
Actin


IP: Sp1
1% Input
300 μM ART:
-
+
No IP
IGG
-
+


CMV-neo
CMV-Sp-1
ART (μM) :
-
+
-
+
Sp1
Cyclin A
Actin

Discussion

Artemisinin and its derivatives have been demonstrated to have antiproliferative effects on multiple cellular and animal models of cancer through a variety of models, many of which not involving the creation of free radicals (33-35). We have demonstrated that Artemisinin and its semi-synthetic derivative Artesunate can inhibit the growth of T-47D human breast cancer cells via induction of G_1 and G_2 cell cycle arrest, with AE being the more potent of the two compounds. This effects was observed to be both dose and time dependent. This arrest is likely due to the ability of both compounds to downregulate the expression of Cyclin A and both of its regulatory partners CDK1 and CDK2. Other critical G_1 and G_2 factors appeared to be unaffected by treatment with the compounds. The regulation of protein expression was driven by phytochemical mediated ablation of mRNA transcripts, and for Cyclin A in particular, ART mediated its promoter activation via disruption of Sp1 activation via a disruption in Sp1 protein levels. Sp1 expression appeared to be necessary to mediate the ART effect on Cyclin A expression because exogenous expression of the transcriptional activator appeared to rescue Cyclin A protein from ART mediated ablation.

The most significant effects of ART and AE on the expression of cell cycle genes is that they both cause a selective decrease in Cyclin A, CDK1, and CDK2. While CDK2 expression has long been implicated with increased chances of breast cancer recurrence and metastasis (36), Cyclin A has recently also been implicated as an effective prognostic marker for ER+ breast cancer for increased, recurrence, and decreased survival, as well as tamoxifen resistance (37). Interestingly though, *decreased* Cyclin A expression both in cells and in tissue samples is linked with *increased* levels of metastasis, through direct activation of RhoA and induction of the epithelial to mesenchymal transition (EMT) (38,39). Cyclin A expression has also been implicated in conferring resistance to therapy in other cancer types, such as cisplatin resistance in endometrial carcinoma, another ER+ cancer sub-type (40). Given these newer data in the literature and its conflicting roles in cancer progression, control over Cyclin A expression becomes even more important than previously thought.

We recently reported that in MCF-7 human breast cancer cells, Artemisinin induced a G_1 cell cycle arrest by disrupting the expression of the transcription factor E2F1, and thereby disrupting its binding to the key G_1 cell cycle regulators CDK2 and Cyclin E, disrupting their expression (41). We are still working to determine if Artemisinin disrupts E2F binding to the Cyclin A promoter, which is known to have two variant binding sites in the 5' region of the gene, and through formation of inhibitory transcriptional complexes has been demonstrated to inhibit expression (42), it is possible that Artemisinin-mediated regulation of these factors could play a role in altering Cyclin A expression. We have also reported the Artemisinin-induced G_1 cell cycle arrest in LNCAP prostate cancer cells was mediated by a downregulation of CDK2 and CDK4, which in turn was induced by a disruption of Sp1 binding to the promoters, through Artemisinin did not have an effect on overall Sp1 expression, instead it mediated changes in phosphorylation status, likely altering co-activator recruitment (25). Interestingly in endometrial cancer cells, Artemisinin did not modulate Sp1 binding to the CDK4 promoter, but instead disrupted CDK4 activation by altering NFκB cellular localization (43) which indicates the activity of ART is likely tissue specific. In osteosarcoma cells, the Artemisinin derivative Artesunate induced apoptosis as well as a G_2/M cell cycle arrest, though the mechanism was not thoroughly studied (44). From these studies, it is obvious that Artemisinin can induce cell cycle

arrest, but the mechanisms of regulation differ based on either the cell or tissue type. Future studies could focus upstream, potentially on the direct binding target(s) of Artemisinin and its derivatives that can give rise to such diverse intracellular effects.

The anti-growth effects of Artemisinin and its derivatives has been demonstrated to be selective for cancerous cells when compared to non-tumorigenic cells lines in a variety of systems. Artemisinin selectively controlled the growth of breast cancer cells compared to a non-tumorogenic cell line (41), leukemic cells when compared to normal primary lymphocytes (45) and ovarian cancer cells compared with normal ovarian epithelial cells (46). These studies make Artemisinin derived compounds even more compelling when it comes to their potential use as an anti-cancer agent. While we have demonstrated its ability to cause growth arrest in an early stage, ER positive breast cancer cell line, further study of its ability to regulate the Cyclin A promoter is needed to definitively say what the critical Artemisinin regulated factors are. In addition, similar studies will need to be carried out with Artsenuate to be sure that the two compounds are acting through the same mechanism in this system as our preliminary data suggests. Future studies will also need to clarify whether Cyclin A is the key to Artemisinin-mediated regulation in early stage breast cancer or if it is the altered expression of CDK1, CDK2, or some combination of the three. Overexpression and interfering RNA studies should be carried out against each of the three cell cycle factors in the presence or absence of ART and AE followed by flow cytometry to achieve this. Nonetheless, this study has demonstrated a novel mechanism in which Artemisinin and Artesunate exert control in a transformed cell line.

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Chapter III

1-Benzyl-I3C Derivatives Slow the Growth of Human Breast Cancer Cells and Melanoma Cells and are More Potent

Abstract

1-Beznyl-Indole-3-Carbinol (1-Benzyl-I3C) is a potent derivative of the phytochemical Indole-3-Carbinol (I3C), which exhibits many anti-cancer properties in many different types of cancer. The potency of 1-Benzyl-I3C has been demonstrated to be much greater than that of I3C by a factor of 100, and by a factor of 1000 in biochemical systems. Two primary target proteins of I3C have recently been discovered, Human Neutrophil Elastase in breast cancer and Neural Precursor Cell Expressed, Developmentally Down-Regulated 4 (NEDD4-1) in melanoma. In this preliminary study, we utilize *in silico* modeling of several 1-Benzyl-I3C derivatives on Elastase or NEDD4-1 to analyze their potential interactions, and we analyze the antiproliferative effects of the derivatives to determine what structural modifications make more potent antiproliferative derivatives in MCF-7 human breast cancer cells and G361 melanoma cells. All derivatives were able to inhibit cell growth, as demonstrated by cell proliferation assays, but none were as potent as the parental 1-Benzyl-I3C. In breast cancer cells 1-phenyl-1*H*-indol-3-yl-carbinol (2160) was the most potent and in the melanoma cells 1-p-tolyl-1*H*-indol-3-yl-carbinol (2242) was. The inhibition of proliferation was due, at least in part, to induction of G_1 cell cycle arrest by all derivatives in both cell types. Since PTEN is a substrate of the ligase NEDD4-1, we tested PTEN expression in the presence of the derivatives and found that PTEN levels were stabilized by all derivatives indicating their potential to disrupt NEDD4-1 function.

Introduction

Human melanoma accounts for only 2-4% of all skin cancers, yet is responsible approximately 80% of skin cancer related deaths, primarily because of its high rates of metastasis (1,2). Over the last decade, several promising therapies have entered the market, however many melanomas remain resistant or develop resistance to these. As a result there is a constant need for the development on new therapies (3). Dietary phytochemicals are a category of promising but largely untapped source of agents that have demonstrated many anticancer properties (4,5). Within this category of anticancer phytochemicals, Indole-3-Carbinol (I3C), a compound derived from the hydrolysis of glucobrassicin found in cruciferous vegetables is of particular interest because of its wide range of anticancer properties. Epidemiological evidence has noted that people with an increased dietary intake of the vegetables containing this compound have a lower overall cancer rate (6,7). In addition, I3C has been demonstrated to induce cell cycle arrest, apoptosis, disrupt migration, and disrupt hormone receptor signaling in multiple cancer sub-types (8-13). The mechanisms that are responsible for the tumor-specific anti-proliferative responses are not yet clearly defined, however many of the responses can be attributed to the ability of I3C to interact with and inhibit specific target proteins that can regulate downstream signaling cascades.

Our lab has identified the only two known direct target proteins of I3C, Human Neutrophil Elastase and NEDD4-1 (14,15). Elastase is a serine protease existing both intracellularly as well as extracellularly in a variety of tissues, whose expression and activity has been determined to be a prognostic marker for late stage, advanced breast cancer with reduced survival in patients (16,17). I3C has been demonstrated to be a non-competitive inhibitor of elastase, which prevents the cleavage of the CD40 member of the tumor necrosis factor receptor gene family, which ultimately leads to a disruption in NFκB nuclear localization and subsequent proliferative transcriptional activity (18,19). Inhibition of elastase also prevents the cleavage of full length Cyclin E to low molecular weight forms whoes presence leads to hyperactive CDK2 and resulting loss of control in the cell cycle (20). Elastase also exists as an extracellular protein which is able to cleave the extracellular matrix protein elastin, making it easier for the cells to migrate and metastasize to other sites (21). In multiple breast cancer models, si-RNA knockdown of Elastase mimics the induction of cell cycle arrest normally triggered by I3C, demonstrating the protein's essential role as an I3C target protein (22).

NEDD4-1 is a HECT class E3 ubiquitin ligase whose function among other things is to target the Tumor Suppressor Phosphotase and Tensin Homolog (PTEN) for proteolytic destruction (23). PTEN is a phosphotase that dephosphorylates phosphatidyl inositol-3,4,5-triphosphate (PIP3) and converts it to phosphatidylinositol 3,4-bisphosphate (PIP2), which prevents PIP3 from interacting with phosphotidylinositol-dependent kinase 1 (PDK1) which normally would phosphorylate and activate AKT, leading to activation of downstream proliferative, survival, and anti-apoptotic pathways (24,25). Activating mutations or overexpression of NEDD4-1 can increase tumor genesis via downregulation of PTEN levels in multiple cancer types including lung, breast, and melanoma (26,27). In addition, overexpression has been implicated as a prognostic marker in gastric cardia adenocarcinoma, as well as other cancerous systems (28). Previous work in our lab has demonstrated through *in vitro* experiments as well as *in silico* modeling that in human melanoma cells, I3C can bind to and inhibit the activity of NEDD4-1, stabilizing PTEN levels and inducing the apoptotic response (15).

The relatively high concentration necessary to induce antiproliferative effects (typically on the order of 200 μM), as well as its ability to dimerize into 3-3'-diindolymethane (DIM) reducing bioavailability are of concern, and spark the need to develop more stable and more potent derivatives that can still bind to and inhibit I3C target proteins. One such derivative has already been studied in both breast cancer and melanoma cells; 1-Benzyl-I3C (19,29). 1-Benzyl-I3C was found to induce similar anti-proliferative effects in cells at 1000-fold smaller concentrations than required for I3C. Given this, there is potential to create other synthetic I3C derivatives that are more potent, robust, and stable then the parental molecule in human melanoma cells. In this study, we utilize *in silico* modeling as well as cell proliferation data and protein expression data in human breast cancer cells and melanoma cells to study the ability and strength in which the I3C derivatives can bind to Human Neutrophil Elastase and NEDD4-1 to induce a cellular response.

Materials and Methods

In Silico Computational Simulations – performed by Jeanne Quirit

The structures of the NEDD4-1 HECT domain and human elastase were obtained from the Protein Data Bank with accession numbers 2XBF and 3Q76, representing the NEDD4-1 HECT catalytic domain and the elastase protein, respectively. The PRODRG server was used to produce the topology files for modeling the I3C, 1-benzyl-I3C, and 1-benzyl-I3C analogs structures. The protein (2XBF and 3Q76) and ligand (indolecarbinol) structures were subsequently loaded into the Hex Protein Docking program. Prior to docking the structures, all water molecules and hetero molecules were manually removed by editing each PDB file. Shape and electrostatics were used as restrictive parameters to model binding between the receptor and ligand. Modeling results were visualized using PyMol. The program LigPlot was then used to generate schematic diagrams that illustrate the pattern of interactions between the 3-D coordinates of the protein and bound ligand.

Cell Culture

MCF-7 human breast cancer cells and G361 human melanoma cells were obtained from American Type Culture Collection (Manassas, VA). MCF-7 cells were grown in DMEM, supplemented with 10% fetal bovine serum (Sacramento, CA), 2mM L-glutamine (Sigma-Aldrich), 10µg/mL insulin, 50 U/ml penicillin, and 50U/mL streptomycin. G361 cells were cultured in McCoy's 5A Modified medium (Lonza, Walkersville, MD) supplemented with 10% Fetal Bovine Serum (Gemini, Elizabeth, NJ), 2 mM *L*-glutamine, 50 U/mL penicillin, and 50 U/mL streptomycin (Sigma). Cells were incubated to subconfluency in a humidified chamber at 37^{o}C degrees containing 5% CO_2. Stock solutions of I3C and all derivatives were dissolved in DMSO DMSO at 1000 times the desired concentration. The compounds were then diluted in media and then applied to the culture plates after washing the cells with phosphate-buffered saline (PBS) (Lonza).

Cell Proliferation Assay

Cells were plated onto 24-well tissue culture plates (Nunc, Denmark) at 70% confluency and treated as indicated in triplicate with DMSO vehicle, Tryptophol, I3C, 1-Benzyl I3C, 2242, 2243, 2244, and 2160 for 48 hours. Inhibition of proliferation was measured using the Dojindo Cell Counting Kit-8 (Rockville, Maryland) as per manufacturer's instructions. Briefly, 50µL of the CCK-8 solution was added to each well along with 450µL of full media and incubated for 2.5 hours. Absorbance was read at 450nm and percent inhibition was calculated by standardizing the average of each treatment triplicate to the average value of the vehicle control.

Flow Cytometry

MCF-7 and G361 cells were plated in triplicate onto six-well culture dishes (NUNC-Fisher) and grown to subconfluence. The cells were then treated with for 48 hours as indicated. The cells were then hypotonically lysed in 300 µL of DNA staining solution (0.5mg/mL propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100. Nuclear fluorescence (585nm) was measured on a Beckman-Coulter EPICS XL instrument with laser output adjusted to deliver 15 megawatts at

488nm. For each sample 10,000 nuclei were analyzed and the percentage of cells in G_1, S, G_2/M phase of the cell cycle was determined by analysis with Multicycle provided by Phoenix Flow Systems in the Cancer Research Laboratory, Flow Cytometry Facility of the University of California, Berkeley.

Western Blotting

After indicated treatments, western blots were performed as indicated previously. Mouse anti-PTEN (Santa Cruz, sc-7974) was diluted 1:200 in Tris-Buffered Saline and Tween 20 (TBST). Mouse anti-Hsp90 from BD Transduction Laboratories (#610418 Franklin Lakes, NJ) and anti-MITF antibodies (Thermo-Fisher, #MA5-14146) 1:500 were diluted 1:1000 and 1:500 in TBST respectively. Immunoreactive proteins were detected after a 1 hour incubation with horseradish peroxidase-conjugated secondary antibodies diluted 3×10^{-4} in 1% nonfat dry milk dissolved in TBST. After 3 subsequent washes of 30 minutes each, blots were incubated with enhanced chemiluminescence reagents from Perkin Elmer Inc. (Waltham MA) for visualization on film.

Results

I3C derivatives are predicted to directly bind to both elastase and NEDD4-1

1-Benzyl-I3C is a small molecule able to confer multiple anti-proliferative effects and directly bind to and inhibit critical cellular proteins in both melanoma and breast cancer (29). Its higher potency when compared to I3C, the molecule from which it was derived, likely stems from the addition of a phenyl group on the nitrogen residue. 1-Benzyl has been predicted to bind to hydrophobic residues, so based on this we created a set of 1-Benzyl-I3C-derivatives with different electron donating functional groups that could potentially decrease the predicted free energy of binding. Ideally, the modifications could still allow similar cellular effects as I3C and 1-Benzyl, but decrease the free energy associated with target protein interactions. These derivatives were synthesized by our collaborators, and the IUPAC names and abbreviations are included in Figure 1.

Utilizing crystal structures obtained from the Protein Data Bank, the I3C derivatives were loaded into the Hex Protein Docking program to determine if it was predicted that the derivatives could bind the target proteins. For Elastase, most of the derivatives' binding sites were similar to those of 1-Beznyl-I3C, away from the active site, and away from the binding site of I3C (Figure 2). Each derivative bound with different predicted free energies with 1-Benzyl-I3C binding with lowest predicted free energy. Unlike what was observed for Elastase, for NEDD4-1 the predicted binding sites of the derivatives were not as tightly clustered, with 1-Benzyl again having the highest binding coefficient.

Figure 1.

Structures of Indole-3-Carbinol, 1-Benzyl-I3C, and analogs.

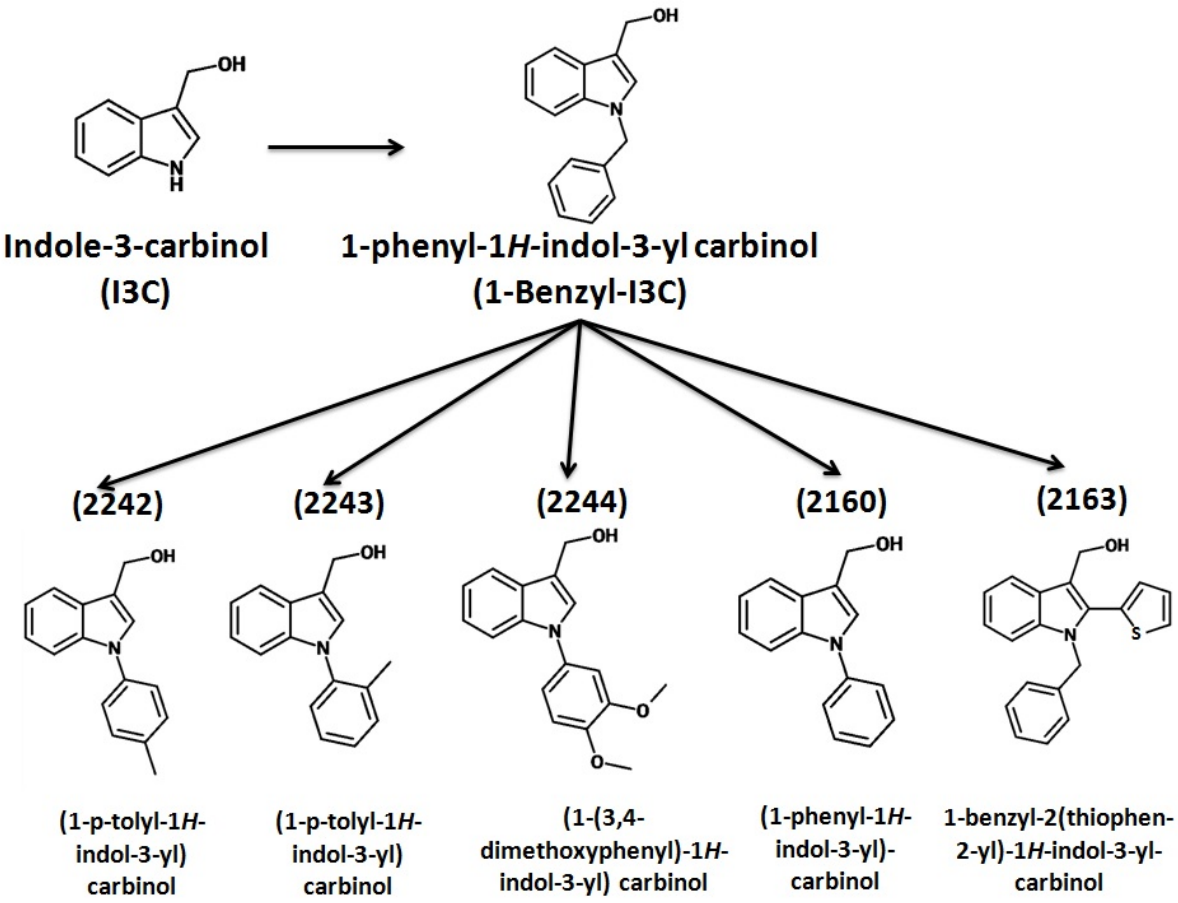
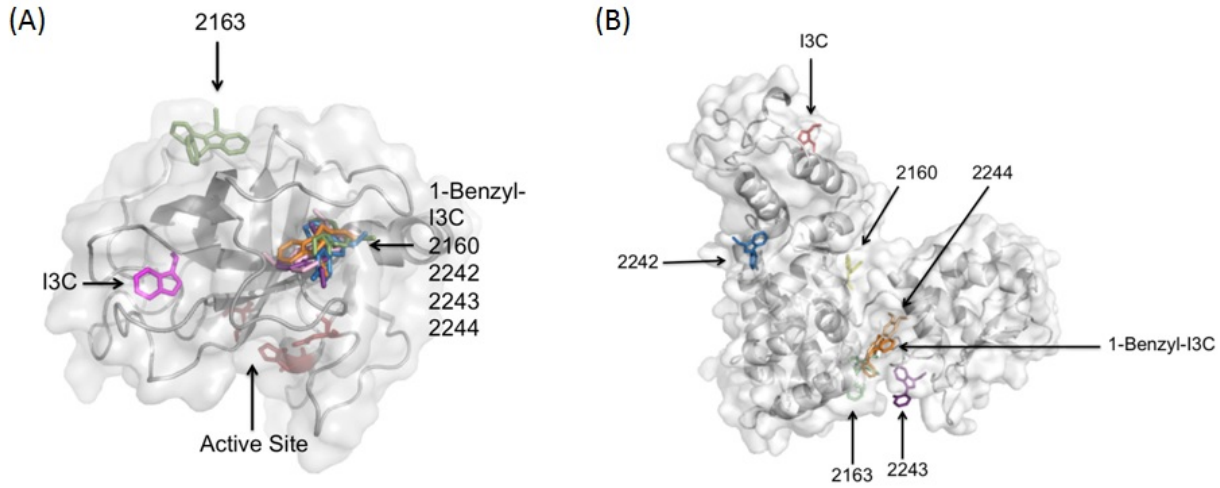

OH
N
H
OH
N
Indole-3-carbinol
(I3C)
1-phenyl-1H-indol-3-yl carbinol
(1-Benzyl-I3C)
(2242)
(2243)
(2244)
(2160)
(2163)
(1-p-tolyl-1H-indol-3-yl) carbinol
(1-p-tolyl-1H-indol-3-yl) carbinol
(1-(3,4-dimethoxyphenyl)-1H-indol-3-yl) carbinol
(1-phenyl-1H-indol-3-yl)-carbinol
1-benzyl-2(thiophen-2-yl)-1H-indol-3-yl-carbinol
S

Figure 2.

In silico computational modeling of predicted interactions of I3C, 1-Benzyl-I3C, compounds 2160, 2163, 2242, 2243, and 2244 with Human Neutrophil Elastase (A) and human NEDD4-1 catalytic HECT domain (B).

The structures of Elastase, NEDD4-1, and the corresponding indolecarbinol compounds were loaded into the Hex Protein Docking program and the computer-aided modeling of binding was performed using shape and electrostatics as restrictive parameters, and visualized using the molecular graphics program PyMol.


(A)
2163
1-Benzyl-
I3C
2160
2242
2243
2244
I3C
Active Site
(B)
I3C
2160
2244
2242
1-Benzyl-I3C
2163
2243

I3C derivatives reduce both MCF-7 and G361 cell number at smaller concentrations than I3C, and induce a G_1 cell cycle arrest

To analyze whether the predicted binding of the derivatives translated into a cellular effect MCF-7 and G361 cells were treated for 48 hours with different doses of I3C, the inactive indole Tryptophol, and the other derivatives or the vehicle control, and relative cell number was quantified (Figure 3). In both cell types, the derivatives were able to reduce cell number better, at lower doses than I3C. In MCF-7 cells, 1-Benzyl-I3C was by far the most potent derivative, reducing cell number by over 60% at a concentration of 1 μM. After 1-Beznyl, the most potent derivatives were 2160, 2244, 2243, and 2242 respectively, all able to reduce cell number by 50% by 50 μM treatment, while the dose of I3C able to initiate such a reduction is between 100 μM and 200 μM. Similar studies were performed in G361 cells in order to analyze whether the predicted binding and possible inhibition of the derivatives to NEDD4-1 could translate to an anti-growth cellular effect (Figure 3). Again, 1-Benzyl-I3C was the most potent derivative, followed by 2242, 2243, 2144, 2160, and 2163 respectively. Like the studies done in breast cancer cells, all derivatives were more potent that I3C, able to reduce cell number by over 50% by 50 μM treatment.

A reduction in cell number can be caused by multiple mechanisms including induction of apoptosis, senescence, or cell cycle arrest. To decipher which of these might be responsible for the reduction in cell number caused by the I3C derivatives, flow cytometry was utilized. Both MCF-7 and G361 cells were treated with the dose of each derivative found to induce the largest reduction in cell number, or the vehicle control. After the treatment, cells were harvested, their DNA stained, and measured by flow cytometry to determine if the derivatives caused any change of cell cycle distribution change (Figure 4). All derivatives induced a G_1 cell cycle arrest in the MCF-7 cells, but interestingly the effect was relatively small. 1-Benzyl-I3C, the derivative able to reduce cell number at the lowest concentration induced an increase in cells arrested in the G_1 phase by 8.5%. The derivative found to trigger the highest increase in G_1 arrest was 2244, with a 12.6% increase. Given the relatively small effect on cell cycle arrest, these derivatives may reduce cell numbers through other mechanisms, many of which can be triggered by inhibition of elastase. Further investigation into how much of a role these other mechanisms play in the reduction in cell number would be an interesting future direction.

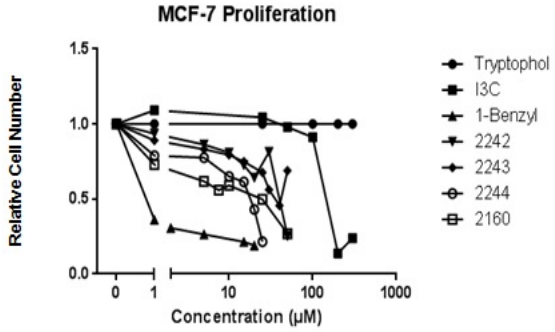
The cell cycle effects in the G361 cells caused by the indole derivatives were much more significant than in the breast cancer cells. Again, the cells were treated with one dose of each derivative found to induce significant cell number reduction. Similar to the cell count data, 1-Benzyl-I3C induced the biggest G_1 arrest when compared to the vehicle control, a 28.1% increase. Similar to the treatments in the breast cancer cells, the potency of the derivatives found in the cell count experiments did not completely correlate with the potency found in the flow cytometry experiments. 2242 induced an 18.2% increase in G_1 arrest, which made it the second most potent derivative in terms of ability to induce arrest. From these data, it is difficult to discern if there are other mechanisms of anti-proliferation that are playing a significant role in the ability of the derivatives to control cell number, but future experiments testing this seem prudent.

Figure 3

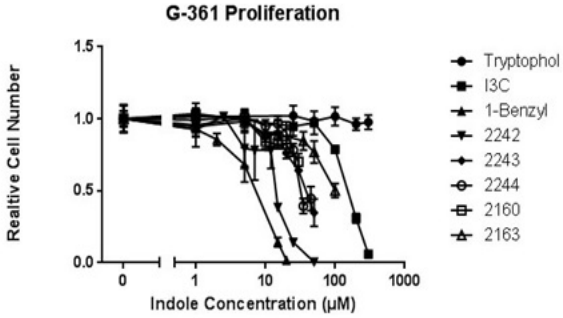
Indole derivatives are able to reduce cell cycle number at concentrations lower than I3C

Cell proliferation assay depicting percent of viable MCF-7 cells (A) or G361 cells (B) at each respective dose after 48 hour treatment. Bottom panel: table listing the half maximal dose of each derivative for the G361 treatment. Half-maximal doses for MCF-7 cells were not calculated

(A)



(B)



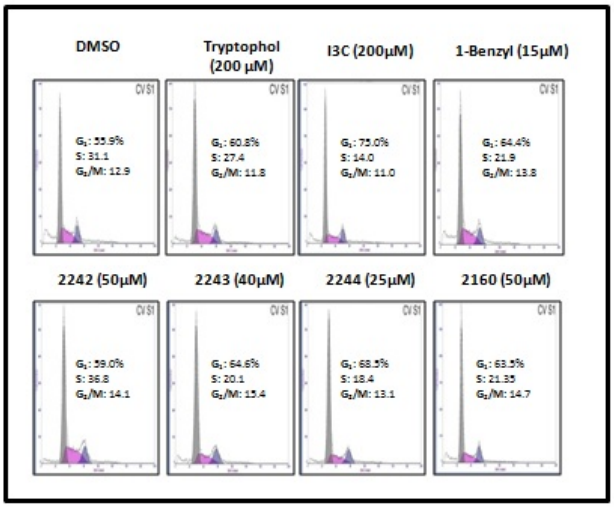
Compound	Structure	Half Maximal Dose After 48 Hours (μM)
I3C		107.3
1-Benzyl-I3C		14.67
2242		18.32
2243		25.06
2244		39.74
2160		47.08
2163		106.15

Figure 4

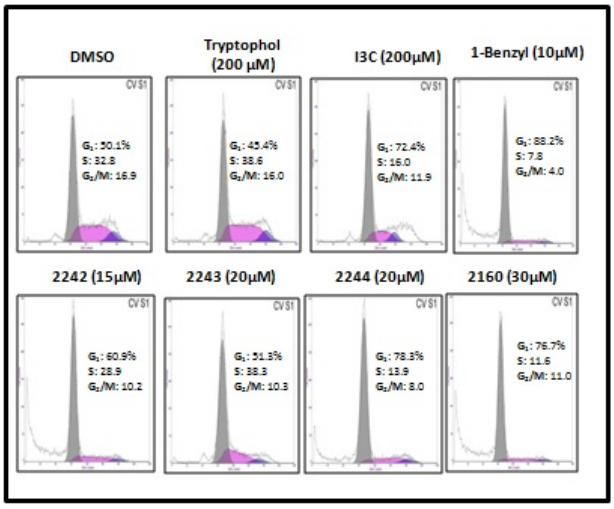
Indole derivatives induce cell cycle arrest in both MCF-7 cells and G361 cells

MCF-7 and G361 cells were treated for 48 hours with the indicated doses of each indole derivative, after which cellular DNA was stained with propidium iodide and measure by flow cytometry. The distribution of the cell cycle population is displayed and percent of population in each phase is indicated. The experiment was performed in triplicate in three independent experiments.

MCF-7


DMSO
Tryptophol (200 µM)
I3C (200µM)
1-Benzyl (15µM)
G1: 55.9%
S: 31.1
G2/M: 12.9
G1: 60.8%
S: 27.4
G2/M: 11.8
G1: 75.0%
S: 14.0
G2/M: 11.0
G1: 64.4%
S: 21.9
G2/M: 13.8
2242 (50µM)
2243 (40µM)
2244 (25µM)
2160 (50µM)
G1: 59.0%
S: 26.8
G2/M: 14.1
G1: 64.6%
S: 20.1
G2/M: 15.4
G1: 68.5%
S: 18.4
G2/M: 13.1
G1: 63.5%
S: 21.35
G2/M: 14.7

G-361


DMSO
Tryptophol (200 µM)
I3C (200µM)
1-Benzyl (10µM)
G1: 50.1%
S: 32.8
G2/M: 16.9
G1: 43.4%
S: 38.6
G2/M: 16.0
G1: 72.4%
S: 16.0
G2/M: 11.9
G1: 88.2%
S: 7.8
G2/M: 4.0
2242 (15µM)
2243 (20µM)
2244 (20µM)
2160 (30µM)
G1: 60.9%
S: 28.9
G2/M: 10.2
G1: 51.3%
S: 38.3
G2/M: 10.3
G1: 78.3%
S: 13.9
G2/M: 8.0
G1: 76.7%
S: 11.6
G2/M: 11.0

In G361 cells, the most potent derivatives upregulate PTEN and MITF-M protein expression levels

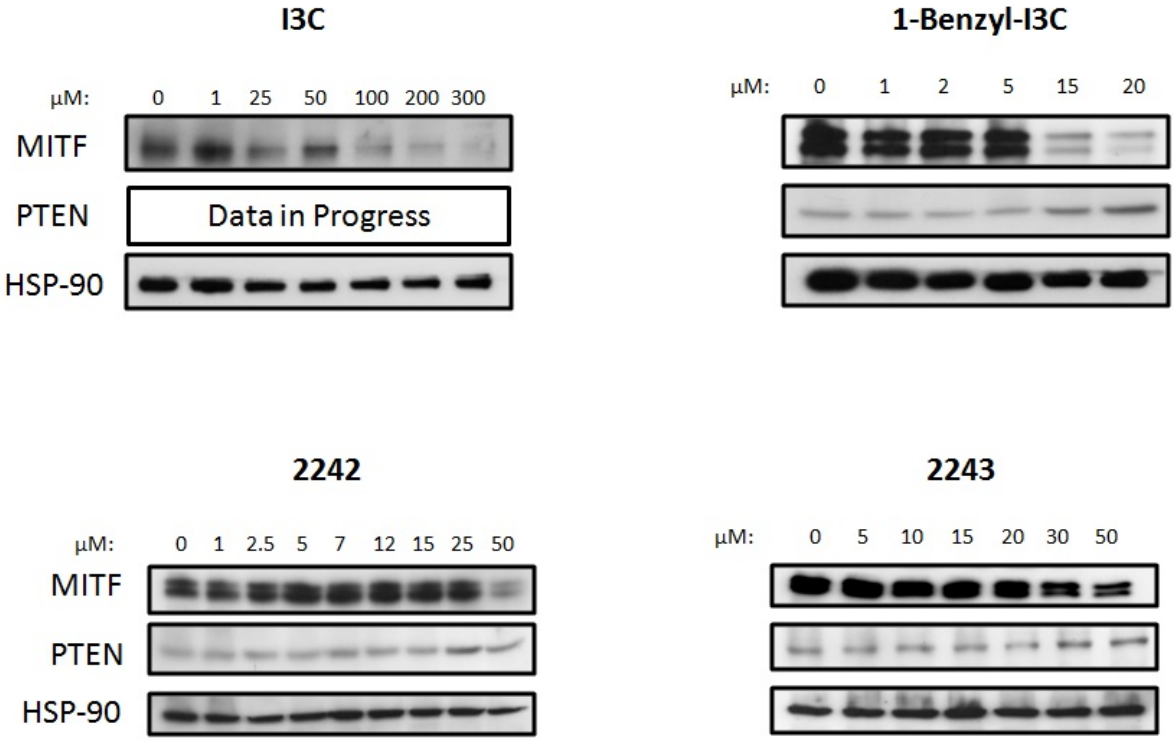
Our lab has already demonstrated that I3C binds and inhibits NEDD4-1 and as a result, potentially stabilizes PTEN levels (15). The *in-silico* modeling performed by Jeanne Quirit predicts that the other I3C derivatives can bind to the ubiquitin ligase as well, possibly inhibiting its activity. To test whether this might be happening, we treated G361 cells for 48 hours with increasing doses of I3C and its three most potent derivatives, 1-Benzyl I3C, 2242, and 2242 (Figure 5), or the vehicle control, and then performed western blotting for PTEN and MITF-M. In each case, PTEN levels increased upon increasing concentrations of the derivatives with the most potent being 1-Benzyl-I3C followed by 2242, and 2243. PTEN is a substrate of the ligase activity of NEDD4-1, and thus PTEN levels are largely under the control of NEDD4-1 activity in human melanoma, so it is likely that inhibition of NEDD4-1 activity by the derivatives is what causes PTEN levels to be stabilized. In previous studies, it was determined that PTEN stabilization by I3C was a post-translational effect, and was not caused by a change in transcript levels (15), and as such we do not anticipate mRNA levels of PTEN to be altered by addition of the derivatives.

In melanoma, MITF-M is at the node of many proliferative processes and exerts control over the cell cycle, survival, and migration among other processes (30-32). Clinically, high expression leads to increased proliferation while lower levels correlate with increased migration and metastasis making MITF-M a difficult protein to characterize (33,34). Because of its obvious importance, we aimed to determine if the I3C derivatives might have other anti-cancer properties in melanoma aside from their potential regulation of NEDD4-1 function. Under the same conditions used when analyzing PTEN protein levels, we immunoblotted for MITF-M expression (Figure 5). All derivatives were found to regulate MITF-M levels, though at different concentrations. I3C began to significantly attenuate expression at 100 μM, while 1-Benzyzl I3C conferred a similar result at 15 μM. Between 2242 and 2243, treatment with 2243 was able to downregulate expression at a lower concentration (30 μM) than 2242 (50 μM), which is a bit surprising since 2242 was more potent in the cell count experiments. Like the earlier results, all of the tested derivatives were more potent than I3C, but none as potent as 1-Benzly-I3C.

Figure 5

Indole derivatives downregulate MITF-M protein levels, and upregulate PTEN levels

G361 cells were treated for 48 hours with increasing doses of I3C, 1-Beznyl-I3C, 2242, or 2243. Cells were lysed, elecrophoretically fractionated and probed for either MITF-M or PTEN. HSP-90 was used as a loading control.


I3C
μM: 0 1 25 50 100 200 300
MITF
PTEN
Data in Progress
HSP-90
1-Benzyl-I3C
μM: 0 1 2 5 15 20
2242
μM: 0 1 2.5 5 7 12 15 25 50
MITF
PTEN
HSP-90
2243
μM: 0 5 10 15 20 30 50

Discussion

In searching for small molecules that demonstrate significant anti-cancer capabilities, the strategy of synthesizing derivatives from a successful parental molecule is widely utilized in the biotechnology sector, pharmaceuticals, and even in academia. Based on initial analysis of the compounds' ability to bind to and alter function of target proteins, newly synthesized derivatives can be made to strengthen those interactions and improve function and potency of the compound. Functional testing must be carried out to determine if changes in predicted binding strength, or predicted ability to alter function correlate with actual intracellular events, and ultimately clinical outcomes. In this preliminary study, we applied functional tests to compounds derived from 1-Benzyl-I3C to determine if they had improved anti-cancer properties after first using *in silico* modeling to predict if they could interact with the target proteins. Data from our study suggests that the derivatives are predicted to bind to both human neutrophil elastase, an enzyme involved with breast cancer, and the E3 ubiquitin ligase NEDD4-1, a critical proto-oncogene in melanoma. NEDD4-1 is a member of the HECT family of ligases, which are implicated in many different diseases including cancer, cardiovascular, immune, and neurological disorders (35). E3 ligases in general are very difficult to target with small molecule therapies, and the HECT family subgroup of ligases is even more difficult. Recent work in our lab has demonstrated that I3C can bind to and inhibit NEDD4-1, which would make it one of the first known inhibitors of that molecule (Quirit et. al. in preparation) though a very recent study has demonstrated the discovery of a different indole as an inhibitor of NEDD4-1 (36). The potential discovery of other compounds with similar capabilities to inhibit NEDD4-1 function is exciting, and could lead to a better understanding of what chemical properties would make an even better inhibitor.

Functionally, we determined that all of the derivatives were able to downregulate cell number in both breast cancer cells and melanoma cells at smaller concentrations than I3C, but higher concentrations than 1-Benzyl I3C. The way in which the compounds were able to achieve this was, at least in part, through induction of G_1 cell cycle arrest where interestingly in melanoma cells, the derivatives were not as potent as 1-Beznyl-I3C, but in breast cancer a few derivatives were indeed more potent. It is possible that in both cells lines, but maybe more so for the MCF-7 cells, that the derivatives are acting through multiple mechanisms to control cell proliferation and survival, and those other effects were not captured via flow cytometry. Further studies should be carried out to determine if indeed this is what is happening since both target proteins, elastase and NEDD4-1, are nodes for multiple proliferative and survival pathways (16,37-39). To indirectly determine if the derivatives were able to directly inhibit NEDD4-1 function, we analyzed PTEN expression levels, a potential substrate of NEDD4-1, after treating the melanoma cells with the derivatives. Stabilizing PTEN levels represents a potential strategy to target melanomas that express wild type forms of the protein (40-42). PTEN is able to reduce levels of activated AKT-1 which controls survival pathways through its activation of MDM2, which tags p53 for destruction, preventing the activation of p53 mediated apoptosis (43-45) . We found that PTEN levels were stabilized in the presence of all of the derivatives, with 1-Benzyl-I3C as the most potent derivative followed by 2242, and 2243, demonstrating the potential for indole compounds as potential therapeutics in melanoma.

Our results suggest that 2242 might be one of the strongest 1-Beznyl-I3C derivatives that can inhibit NEDD4-1 function. While direct binding and inhibition assays are currently being

carried out, we can speculate as to why this compound is able to confer stronger intracellular effects than the others. The binding pocket that 2242 is predicted to interact with contains many residues that are hydrophobic and basic in nature (data not shown). The additional functional group on 2242 increases pi stacking interactions as well as charge charge interactions between the compound and the pocket which contain multiple tyrosine residues and other basic residues as well, while the predicted binding pockets of the other derivatives do not contain such residues. This model needs to be tested, and indeed future experiments include mutagenesis of those tyrosine residues to others that are not polar or basic to see if intracellular function of 2242 is disrupted. These future studies will help understand what modifications would be most beneficial to making even stronger derivatives in the future.

It was intriguing to see that I3C, 1-Beznyl-I3C, 2242, and 2243 were all able to modulate MITF-M expression in melanoma cells. MITF-M is often referred to as the "master regulator" of melanocyte development and melanoma progression (46). It is able to control many functions in melanoma from cell cycle progression, migration, survival, and differentiation, its function in melanoma is extremely complex and still under debate. The new understanding of how MTIF-M works and what it's downstream targets are reveals new therapeutic strategies base around the suppression of this protein, even though it has a hard protein to directly target with small molecules (46). We have demonstrated the ability of small indole derivatives to exert control over expression at concentrations below that the parental compound, I3C, has been demonstrated to be tolerated in humans (47,48).

In this study, we have demonstrated anti-proliferative properties of 1-Benzy-I3C derivatives in human breast cancer cells and melanoma. The compounds are potentially directly interacting with either Human Neutrophil Elastase in the breast cancer cells, or NEDD4-1 in the melanoma cells disrupting their function. While this study is still preliminary, it encourages further study of these compounds to verify the predicted binding sites of these derivatives on target proteins through further modeling and site-directed mutagenesis. Also, *in-vitro* testing of the enzymatic activity of both enzymes should be tested to verify that predicted binding indeed alters enzymatic function.

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Conclusion and Future Directions

Melanoma is a complex and dangerous disease, mainly because typically late detection and its ability to metastasize to distant sites. Melanoma presents a challenge compared to other cancers because it is not hormone dependent, and as a result entire classes of therapeutics cannot be utilized. Other classes of targeted therapeutics such as BRAF inhibitors such as vemurafinib and MEK inhibitors are utilized with varying degrees of success, though acquisition of resistance after induction of the therapy is common. Immunotherapies such as ipilimumab and anti-programmed cell death 1 have demonstrated promising results in a small number of patients, and there is not yet a way to predict who will respond to the therapies. This makes characterizing melanoma phenotypes and expression profiles critical so patterns of expression and responsiveness can be discovered. Combination therapies are an area of active exploration, since they are more likely to overcome potential acquired resistance.

The role of aspirin in the progression of melanoma is a relatively new topic of exploration. After the demonstrated success of long-term aspirin intake in decreasing colorectal cancer incidence and mortality, aspirin's anti-cancer abilities in other phenotypes has become an area of active exploration. Longitudinal clinical studies of low-dose, log-term aspirin intake in melanoma have indicated aspirin can be beneficial by decreasing rates and mortality, though the literature is more limited when compared to colorectal studies. In these systems, the exact mechanism of action in which aspirin mediates its effects is relatively unknown or explained mostly through its ability to inhibit the activity of COX2. Furthermore, there have been very few studies that focus on acute treatment of melanoma with high dose aspirin and how those effects differ from longitudinal studies.

Concerns about gastrointestinal bleeding and other related issues have impeded the study of higher doses of aspirin as a potential anti-cancer agent. We combined aspirin and Indole-3-Carbinol to study the effects of the combination on the growth of melanoma cells. We found that the combination of the two compounds conferred greater inhibitory results than either of the two alone, and the effects was even greater than the sum of the effects of the two compounds alone, indicating that the combination is acting synergistically upon the cells. These effects were likely mediated through disruption of MITF-M signaling. The implications of this are that clinically, aspirin could be applied in smaller doses in combination with I3C to achieve greater effects than aspirin treatment alone, helping circumvent concerns about GI bleeding. To further investigate the potential of this combination, mouse studies should be carried out utilizing tumor xenografts of human melanoma cells and different combinations of I3C and aspirin. Since both compounds are tolerated in high doses in mice, it would be interesting to see if the already noted effects of aspirin on tumor growth are amplified with combination treatment. The result of downregulated MITF-M in an *in vivo* system is immediately clear since extremely low levels have been implicated with increased migration and metastasis.

aspirin treatment has been implicated to inhibit invasiveness and migration in multiple cancer systems via inhibition of the Nuclear factor-kappa B (NFκB) pathway, but this has not yet been demonstrated in melanoma. It has also been noted to inhibit these processes via inhibition of Matrix Metalloproteinase-2 and 9 (MMP-2 and 9) in liver cancer cells and in macrophages, enzymes that are critical for breaking down the ECM and heavily associated with migration. I3C has also been demonstrated to regulate both migration and invasion in breast cancer, melanoma

and other neoplasias through a variety of mechanisms including the downregulation of MMP-2 and 9, and the disruption of cell adhesion through upregulation of E-Cadherin. It would be worthwhile to investigate if the two compounds produce synergistic inhibition of these properties both *in vitro* and *in vivo*, and if they are affecting the same mechanism, thus counteracting any pro-migratory processes that are potentially activated as a result of the downregulation of MITF-M expression.

The phytochemical Artemisinin and its derivatives has been one of the best anti-malarial agents over the last 2 decades. Its anti-parasitic activity is attributed to the creation of ROS in the parasitic cells, and this mechanism has been implicated for some of its anti-cancer abilities as well. In Chapter II of this thesis, we elucidate how Artemisinin and its derivative Artesunate are able to transcriptionally dowregulate Cyclin A2, CDK1, and CDK2, critical cell cycle factors that exert control over the G_1/M and G_2 phases of the cell cycle. While Artesunate was demonstrated to be more potent than the parental molecule, we determined that mechanistically, a decrease Sp1 expression and thus binding to the Cyclin A promoter initiated by Artemisinin treatment was how Artemisinin exerted control on Cyclin A expression. Presently, we do not know how Sp1 levels are regulated by Artemisinin and this would be an interesting area of study. Our lab and others have demonstrated Artemisinin exerts control in breast cancer cells partially through regulating Estrogen Receptor Alpha expression, and it would be worthwhile to study the effects on Cyclin A, CDK1, CDK2, and Sp1 expression in a hormone null system to see if these effects are dependent on that regulation. In other systems, Sp1 levels are unaltered by Artemisinin treatment, though its phosphorylation state is, altering its ability as an activator. Future study should focus on this potential tissue specific effect of how Artemisinin controls Sp1 levels and if the derivative Artesunate can do the same in lower doses. Sp1 phosphorylation status can be altered by CDK2, and CDK2 has 2 Sp1 sites in the proximal region of the promoter, kinetic study of Sp1 phosphorylation status and resulting activating ability would help decipher if CDK2 expression were altered first or Sp1 phosphorylation via other mechanisms. Additionally, the effects of other Artemisinin derivatives such as Dihydroartemisinin should be explored in breast cancer cells to analyze if they act through similar mechanisms.

Direct target interactions between small molecules and their target proteins are critical in initiating downstream physiological effects. In Chapter III, we studied the physiological effects of multiple 1-Benzyl-Indol-3-Carbinol (1-Beznyl-I3C) derivatives in breast cancer and melanoma cells and their potential ability to interact with Human Neutrophil Elastase and NEDD4-1. We found that 1-Benzyl-I3C was the most potent derivative; it significantly attenuated cell number in both systems, initiated significant cell cycle arrest, and stabilized PTEN (a critical downstream factor of NEDD4-1) levels at the lowest doses. After that, we found that the derivative 2242 was the next most potent molecule. While these studies are very preliminary, the next step would be to analyze the structure of these compounds and their predicted free energy binding to target proteins, and compare that to proliferative effects observed. From this analysis, it might be possible to predict what added functional groups could increase binding strength and ideally potency. Site directed mutagenesis is currently being carried out directed at the predicted binding sites of the target proteins to functionally test the *in silico* predictions.

This thesis has demonstrated that small molecule phytochemicals and their derivatives represent a class of compounds with potential clinical relevance. While I3C has been studies extensively

as an anti-cancer agent, aspirin has not, and the combination has never been shown to have synergistic effects on critical melanoma genes. Combination therapies have many advantages over mono-therapies and this result should encourage more study of the combination and even aspirin alone as a possible anticancer treatment. Because of the use of Artemisinin and its derivatives in other clinical areas, its safety in humans has been demonstrated and these studies have demonstrated the potential as anti-cancer treatment that could encourage clinical testing. Finally, by derivatizing 1-Beznzyl-I3C, there is potential to make even more potent compounds with very specific target protein interactions, which would decrease dose needed to achieve anticancer effects.