Anticancer mechanism of action and therapeutic potential of the natural phytochemical Indole-3-Carbinol and its potent synthetic derivative1-benzyl Indole-3-carbinol in Human Melanoma.

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Abstract

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Indole-3-carbinol (I3C), a key bioactive product of Glucobrassicin, found in cruciferous vegetables has been demonstrated to control the formation, growth and metastasis of certain human cancers with minimal side effects especially during prolonged treatments. It is currently in clinical trials for the prevention and treatment of reproductive cancers such as breast and prostrate cancer. However, relatively little is known about the effects of I3C on human skin cancer.

Human melanoma, the most aggressive form of skin cancer currently poses a big clinical challenge due to the inevitable development of resistance to currently available therapies, and has a dismal 5year survival rate. Approximately 50-60% of melanomas contain an oncogenic mutation in the B-RAF gene that leads to constitutive activation of its downstream proliferative signaling via the MAP kinase pathway. One therapeutic strategy that showed significant promise in preclinical and clinical trials was the selective targeting of the mutant BRAF in melanoma by synthetic B-RAF inhibitors like Vemurafenib or Dabrafenib. Unfortunately the long-term prognosis of metastatic melanoma patients treated with these oncogenic BRAF inhibitors remains poor due to the emergence of early or acquired resistance to these drugs and an increased risk for cancerous or precancerous non-melanoma skin lesions.

Efforts to unravel the molecular mechanisms leading to development of resistance to BRAF inhibitors have uncovered a complex network of signal transduction pathways that get activated and cross talk with each other to circumvent the inhibition of the BRAF signaling pathway. Some of the reported mechanisms of acquisition of BRAF inhibitor resistance include the homozygous loss of the tumor suppressor protein PTEN, a compensatory amplification of the lineage specific master regulator of melanoma biology Micropthalmia Associated Transcription

Factor–Isoform M (MITF-M), as well as elevated levels of the Wnt proliferative signaling pathway. Therefore there is an emerging need in the field of melanoma-targeted therapy for drugs either with multiple mechanisms of actions which, can inhibit more than one of these oncogenic pathways or molecules that can be effectively combined with clinically used drugs to bring about a greater antiproliferative response with minimal levels of toxicity.

In this context, I3C was found to induce significant G_1-phase cell-cycle arrest and apoptosis by stabilization of PTEN in human melanoma cells that express wild-type PTEN. This effect was not observed in cells with mutant or null PTEN genotypes neither did I3C have any detrimental affect on normal human epidermal melanocytes. In wild-type PTEN-expressing melanoma cell xenografted tumors formed in athymic nude mice, I3C attenuated growth of the tumors in vivo and the residual tumors showed increased levels of PTEN protein. Mechanistically, I3C disrupted the ubiquitination of PTEN by NEDD4-1, which prevented the proteasome-mediated degradation of PTEN without altering its transcript levels. RNAi-mediated knockdown of PTEN prevented the I3C-induced apoptotic response, whereas knockdown of NEDD4-1 mimicked the I3C apoptotic response, stabilized PTEN protein levels, and downregulated phosphorylated AKT-1 levels. Co-knockdown of PTEN and NEDD4-1 revealed that I3C- regulated apoptotic signaling through NEDD4-1 requires the presence of the wild-type PTEN protein. Finally, in silico structural modeling, in combination with isothermal titration calorimetry analysis, demonstrated that I3C directly interacts with purified NEDD4-1 protein. This study for the first time identified NEDD4-1 as a direct binding target of I3C in human melanoma.

However the antiproliferative action of I3C was found to be not restricted to melanoma cells lines expressing wild-type PTEN only. Oncogenic BRAF-V600E expressing melanoma cells were sensitive to I3C too, both in cell culture and in-vivo, independent of the status of PTEN. However in cell lines with a mutant BRAF and wild type PTEN the antiproliferative effect of I3C was found to be the maximum. In contrast, wild type BRAF-expressing melanoma cells remained relatively insensitive to I3C anti-proliferative signaling. In BRAF-V600E-expressing cells as well as in vivo, I3C strongly inhibited phosphorylation of the downstream effectors of BRAF signaling namely MEK, MAPK, and downregulated protein levels of BRN2, the transcription factor that mediates control of the BRAF signaling pathway over the melanocyte master regulator - MITF-M. Consequently upon treatment with I3C, MITF-M protein and transcripts levels were strongly attenuated with a significant inhibition of promoter activity and loss of endogenous BRN-2 binding to the MITF-M promoter. In silico modeling using known crystallographic structures of BRAF proteins predicted a thermodynamically stable I3C binding site within the HRD motif in the BRAF-V600E catalytic loop, whereas only low affinity interactions outside the catalytic domain were predicted with the wild type BRAF. In vitro kinase assays using immunoprecipitated BRAF-V600E and wild type BRAF demonstrated that I3C directly and selectively inhibited the enzymatic activity of only the oncogenic BRAF but not of the wild type protein.

Compared to the effects of each compound alone, combinations of I3C and the clinically used BRAF-V600E inhibitor Vemurafenib cooperatively inhibited melanoma cell proliferation and reduced MITF-M levels in BRAF-V600E expressing melanoma cells. Taken together, these results identified oncogenic BRAF-V600E as a key cellular target of I3C in human melanoma. Overall these studies revealed I3C as a rare small molecule with mechanisms of action targeting

two key oncogenic signaling pathways in melanoma and thereby a potential candidate for novel single or combinatorial therapies to treat human melanoma in the clinic.

1-benzyl I3C, a synthetic analog previously demonstrated to be approximately 1000-fold more potent in its antiproliferative effect in human breast cancer cells in –vitro, was tested in an endeavor to find better, more potent anti-cancer compounds against melanoma. 1-benzyl I3C induced an antiproliferative effect and a G1 cell cycle arrest in melanoma cells in culture and attenuated xenografted tumor growth in-vivo in a murine model of melanoma, at 10 fold lower doses, at concentrations similar to currently used melanoma drugs. Additionally, 1-benzyl- I3C was effective against melanoma cells with a wider range of mutation profiles than I3C. Similar to I3C, 1-benyzl I3C significantly downregulated protein and transcript levels of MITF-M, but through the Wnt>GSK3β>β-catenin pathway which, is reportedly activated in one-third human melanoma specimens. 1-benzyl I3C downregulated protein levels of the Wnt co-receptor LRP6 leading to downstream release of inhibition on the β-catenin inhibitory complex, and consequent upregulation of one of the inhibitory proteins GSK3β, causing decrease in both cytoplasmic and nuclear β-catenin. Less nuclear β-catenin resulted in attenuation of MITF-M promoter activity accounting for the observed downregulation of MITF-M transcripts upon treatment with 1-benzyl I3C. Top Flash assays confirmed the involvement of the Wnt>GSK3β>β-catenin pathway in 1-benzyl Indole-3-carbinol's mechanism of action. Combinations of 1-benzyl I3C with Vemurafenib used to treat melanoma cells, displayed a cooperative antiproliferative effect with a concomitant downregulation of MITF-M transcripts greater than each compound alone. This study identified 1-benzyl I3C as a novel synthetic inhibitor of Wnt signaling in melanoma that could be a promising mono or combinatorial therapeutic agent for human melanoma.

This work is dedicated to

My Ma and Papa for dreaming the PhD dream for me,
My Tapu for being my special third parent and blanketing me in tender love and
My Husbendosaur for sharing and pushing this dream to fulfillment.

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INTRODUCTION

Melanoma – A threat on the rise

The incidence of skin cancer over the past three decades have been more than all other cancers combined [1]. It is estimated that one in five Americans will develop skin cancer in the course of their lifetime. Melanoma, a malignant form of skin cancer originating in the melanin containing melanocytes, accounts for less than 2 percent of all diagnosed skin cancer cases, but causes 77% of all skin cancer deaths [1]. Death resulting from melanoma has an alarming rate of one every 57 minutes [1]. The American Cancer Society estimates about 73,870 new cases of invasive melanoma will be diagnosed in the US in 2015 and an estimated 9,940 people will die from it [2]. Among the seven most common cancers in the US, melanoma is the only one whose incidence rate is on a continuous rise [3]. The overall 5-year survival rate for patients is dependent on the stage of detection and outcome is dismal if diagnosis happens after the disease has metastasized to distant organs.

Several epidemiological studies have implicated exposure to ultraviolet (UV) solar radiation as a major etiological melanoma risk factor, with the highest risk factor associated with intermittent sunburns especially during childhood. [4,5]. Ultraviolet radiation from sunlight reaching the earth is composed of 90-99% UVA and only 1-10% UVB. The latter is mostly absorbed by the stratospheric ozone layer [6], which has been adversely affected by modern climate change and the holes in the ozone layer. The high energy UVB radiation is strongly absorbed by the DNA in the epidermal cells causing direct DNA damage by generating two majorly mutagenic photoproducts namely cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), [6,7]. These photoproducts are exclusively repaired by nucleotide excision and loss of this repair system resulting from transformed melanocytes strongly correlate with increased risk of melanoma [6,8] UVA on the other hand induces indirect photo-oxidative DNA damage by generating CPDs and 8-oxodG.

Melanin, the pigment imparting color to human skin is specifically synthesized within melanocytes and has been traditionally implicated to have a protective effect against direct DNA damage by absorbing the energy from UV radiation [9]. However recent evidence from two independent groups suggest that melanin potentially contribute to additional DNA damage, which might increase the risk of skin cancers. UVA causes melanin–dependent melanomagenesis through indirect photo-oxidative DNA damage by generating dark cyclobutane pyrimidine dimers (CPDs), as a delayed response [10,11]. Hence melanin in this case acts as a molecular vector in promoting melanoma.

The race most vulnerable to skin cancer is white Caucasians especially those of Celtic descent living in areas of high ambient UV radiation. The United Nations Environment Program projects a serious increase in skin cancer incidence due to stratosphere ozone depletion. [12]. Based on the current trend scientists have modeled future ozone levels and UVR exposure, which predicts a 10% increase in skin cancer incidence by 2050. Further culturally based behavioral changes have led to much higher UV exposure through tanning beds and sun-bathing. Hence a

combination of genetic predisposition, post-migration geographical vulnerability and modern behavior patterns has in combination resulted in a significant increase in melanoma in the western populations.

The increasing incidence, high mortality and high recurrence of malignant melanoma over the past decades pose an important clinical challenge for the development of novel therapeutics that can target the complexity of the underlying molecular signaling and effectively treat this disease with minimal adverse side effects and recurrence.

Current Treatment Options and the need for better

Five major treatment options are currently available for melanoma as listed by the National Cancer Institute. 1. Surgery of primary tumor and/or sites of metastasis, 2. Chemotherapy 3. Radiation therapy 4.Biologic/Immunotherapy in which the patient's own immune system is restored, boosted or directed to launch a response specifically against the cancer cells 5.Targeted therapy in which drugs or other compounds are used to attack cancer cells. This treatment strategy usually causes less damage to normal cells than chemotherapy or radiation therapy.

Currently used targeted therapies can be broadly classified into the following categories:

- **Signal Transduction Inhibitor therapy**: Uses compounds to selectively target and block specific oncogenic signal transduction pathways to eliminate cancer cells. Examples of currently used Signal Transduction Inhibitors in the clinic to treat melanoma are Vemurafenib/Zelboraf (Roche), and Dabrafenib/Tafinlar (GlaxoSmithKline), both of which are oncogenic BRAF inhibitors while Trametinib/Mekinist (GlaxoSmithKline) is an inhibitor of MEK. All the three drugs inhibit the BRAF signal transduction pathway, which has been implicated in more than 80% of melanoma cases.

- **Oncolytic Virus Therapy:** Uses selective targeting of cancer cells by oncolytic viruses.

- **Monoclonal Antibody Therapy:** Uses monoclonal antibodies designed against surface markers expressed specifically on cancer cells. They are administered alone or conjugated with drugs or toxins.

- **Angiogenesis inhibitors**: Uses compounds to inhibit neoangiogenesis in tumors and thereby attenuate growth**.**

Indole-3-carbinol against Cancer.

Natural dietary phytochemicals represent a promising but largely untapped pool of biologically active chemotherapeutic agents that have the potential to control the formation, growth, and metastasis of human cancers with minimal side effects, especially during prolonged treatments [13–15]. Epidemiological studies have revealed a positive association between consumption of diets rich in Brassica genus vegetables and lower rates of several types of cancers in humans including breast, prostrate, lung and skin cancer [14,15]. Cellular, physiologic, and epidemiologic studies have implicated indole-3-carbinol (I3C), a hydrolysis product of glucobrassicin found in cruciferous vegetables of the Brassica genus, including cabbage, broccoli, and Brussels sprouts, and its natural diindole condensation product 3,3'-diindolylmethane (DIM) as promising anticancer phytochemicals with negligible toxicity [15-19]. Many of these antiproliferative responses are selectively controlled by I3C-activated pathways that are unaffected by DIM. Depending on the human cancer cell type, I3C triggered cell-cycle arrest, apoptosis, and disruption of cell migration, and modulated hormone receptor signaling is mediated by the selective regulation of transcriptional, metabolic, and cell signaling cascades [19-27; reviewed in refs. 15–18]. Effectiveness of I3C's anti-tumorigenic properties has been further confirmed by a panel of short term bio-assays applied to a broad spectrum of phytochemicals, where I3C emerged as one of the few able to negatively regulate carcinogen induced tumor initiation and promotion, DNA damage and oxidative stress [16]. Indole-3-Carbinol has also been tested in clinical trials for prevention and treatment of breast and prostrate cancer respectively [Clinicaltrials.gov Identifier: NCT00033345, NCT00607932, NCT00579332].

A new concept emerging from our ongoing studies is that the presence of specific I3C target proteins that are exclusively expressed in different human cancer cells, mediates the efficacy by which I3C selectively stimulates distinct anti-proliferative signaling cascades in human cancers [18-23]. We have identified two such I3C target proteins. In human breast cancer cells we were the first to establish that I3C and its more potent and stable derivative 1-benzyl-I3C act as direct noncompetitive inhibitors of the proteolytic activity of neutrophil elastase that cause a cellular shift from cell survival to apoptotic signaling [28,30]. The inhibition of elastase enzymatic activity directly prevents cleavage of the CD40 member of the tumor necrosis factor receptor gene family, which causes CD40 signaling to switch from activating cell survival pathways to triggering antiproliferative cascades in human breast cancer cells [29, 30].

Relatively little is known about the effects of I3C on skin cancer cells. I3C has been previously shown to sensitize several melanoma cell lines to UV treatment induced apoptosis and enhance cytotoxic responses in human melanoma [31, 32] and squamous cell carcinomas respectively [33]. Also, ectopic application of I3C directly has been shown to inhibit skin tumor formation in mouse models [34]. However the ability of I3C to induce apoptosis in melanoma cells without concomitant UV treatment has not been previously investigated, and the molecular pathways involved in I3C's mechanism action are unknown. Conceivably, the sensitivity of human melanoma cells to I3C could be to due to the presence of melanoma specific indolecarbinol target proteins that can activate antiproliferative signaling cascades and/or disrupt cell survival pathways.

Developmental pathways activated in melanocytes and melanoma.

PTEN signaling in normal melanocytes

Phosphatase and tensin homolog (PTEN) is a dual specificity phosphatase (DSP) that has both lipid and protein phosphatase function. As a lipid phosphatase PTEN acts on Phosphalidyl Inositol 3-Phosphate (PIP3), resulting in shutting off phosphorylation of AKT and signaling through the PI3K-AKT-mTOR proliferative pathway resulting in a tumor suppressor effect [35] As a protein phosphatase PTEN plays a role in cell cycle regulation and inhibition of cell invasion. The PTEN gene has 9 exons and the protein has the (I/V)-H-C-X-A-G-X-X-R motif characteristic of DSPs. However the active site pocket is deep and wide with two basic residues (Lys125 and Lys128) indicating a preference towards PIP3 and highly acidic residues in polypeptides [36].

As a phosphatase, PTEN acts to remove phosphate groups rendering the substrate protein or lipid either active or inactive depending on the signaling context. The best described substrate of PTEN is PIP3, which the former dephosphorylates to form PIP2. This effect counters the effect of Phosphoinositide 3' kinase (PI3K), which adds a phosphate group to PIP2 to generate PIP3. This membrane bound PIP3 recruits the kinase - phosphoinositide–dependant kinase1 (PDK1) to the cell membrane, via its pleckstrin homology domain, which triggers its activation. Additionally accumulation of PDK1 creates a docking site for AKT on the plasma membrane, which also binds to PIP3 via its pleckstrin homology domain to undergo a conformational change exposing critical Thr 308 residue in the activation loop. PDK1 phosphorylates this residue while PDK2 subsequently phosphorylates Ser 473 on AKT resulting in full-length activation of the protein. AKT subsequently triggers a phosphorylation cascade of downstream target proteins leading to cell-cycle progression and regulation of apoptosis. PTEN acts as a negative regulator of this pro-proliferative signaling cascade by preventing the localization of proteins with the pleckstrin homology domains namely PDK1 and AKT to the cell membrane. One study showed missense mutation in PTEN, PTEN-G129E, which is associated with Cowden disease, specifically disrupts PTEN's ability to recognize inositol phospholipids as a substrate, indicating loss of the lipid phosphatase activity in the etiology of the disease [37].

There are multiple mechanisms of regulation of PTEN including transcription, mRNA stability, microRNA targeting, translation and protein stability and dysregulation of PTEN have been associated with a number of human cancers. For instance PTEN is transcriptionally silenced by promoter methylation in a reproductive cancers like breast, ovarian, endometrial and prostrate cancers [38]. PTEN has also been shown to be post-translationally regulated by ubiquitilation, acetylation, oxidation, phosphorylation and proteasomal degradation *in vitro*. Lys13 and Lys 289 in the PTEN protein are monoubiquitinated, which leads to nuclear import and tumor suppression in vitro. [39]. Polyubiquitination of PTEN by the E3 ubiquitin ligase NEDD4-1 signals proteasomal degradation of PTEN. Gene dosing is yet another layer of regulation of PTEN biology which was found to determine tumorigenic outcomes. Mutations leading to loss of function or reduced levels of functional PTEN protein as well as allelic or total deletions or alterations have been reported in many types of human reproductive cancers as well as melanoma indicating the importance of its role as a tumor suppressor protein [40].

PTEN – the tumor suppressor in melanoma

10-30% melanomas exhibit a loss of PTEN function due to microdeletions, frameshift mutations or epigenetic mechanism [41-43]. The role of PTEN as a tumor suppressor in melanoma was evident from the high frequency of these samples displaying loss of heterozygosity (LOH) of chromosome 10q23, which codes for PTEN. Among the mutations reported in melanoma cell lines and biopsy samples three common mutations emerged and a mutational hot spot was found in exon5, which encodes the phosphatase activity domain. Loss of PTEN expression or alteration of PTEN function in tumor cells therefore result in constitutive activation of pro-proliferative signaling through high levels of active phospho- AKT. Functional studies involving expression of wild-type or substrate-trapping forms of PTEN in HEK293 cells altered the levels of the phospholipid products of PI3K and ectopic expression of PTEN in PTEN-deficient tumor cell lines resulted in the inhibition of protein kinase (PK) B/Akt and regulation of cell survival confirming the tumor suppressor role of PTEN in melanoma [44].

Observations from multiple studies have indicated the existence of collaborative event between BRAF V600 mutation and hyperactivation of PI3K/AKT/mTOR signaling from loss or inactivation of PTEN in melanoma genesis. Functional studies have corroborated these findings [45, 46]. Although largely mutually exclusive from NRAS mutation, PTEN inactivation has been found to non-randomly co-exist with BRAF constitutive activation as well as with wild type BRAF/NRAS melanomas. Furthermore genetically engineered mouse models develop melanoma with 100% incidence only upon the concurrent loss of PTEN and mutation in BRAF [47]. There are several reports indicating that the loss of PTEN reduces the sensitivity of human melanoma cell lines to BRAF/MEK inhibitors by disrupting the induction of apoptosis. [36,48-51]. Decreased PTEN copy number has also been associated with shorter progression free survival in patients treated with the BRAF inhibitor Dabrafenib and higher PTEN protein expression levels were reported to correlate with higher sensitivity to yet another BRAF inhibitor Vemurafenib [52,53] Additionally one of the reported mechanisms of acquired drug resistance in melanoma to BRAF/MEK inhibitor treatment has been inactivation of PTEN resulting in hyperactivity of the PI3K-AKT pathway to restore oncogenic signaling. This provides a strong rationale to assess the clinical benefit of targeting the PTEN/ AKT pathway in melanoma.

Chapter 2 of this dissertation showcases Indole-3-carbinol as an effective inhibitor of PI3K/AKT signaling in melanoma both in cell culture and *in-vivo* in a xenografted murine model. Indole-3-carbinol directly inhibits the function of NEDD4-1, the PTEN specific ubiquitin ligase leading to stabilization of the tumor suppressor protein resulting in an anti-cancer effect. Results of this study in conjunction with the study presented in chapter1 of this dissertation is a first in class preclinical report of a single small molecule directly targeting multiple oncogenic pathways in human melanoma with minimum toxicity. This presents the exciting potential for further clinical development of this molecule to treat melanoma in human patients in the future.

BRAF signaling in normal melanocytes

BRAF is a member of the RAF family of serine/threonine protein kinases encoded on chromosome 7q34. This family consists of 3 kinases, ARAF, CRAF and BRAF. BRAF functions to regulate the MAPK/ERK pathway, a pathway that is conserved in all eukaryotes [54]. The RAS/RAF/MEK/ERK pathway acts as a signal transducer between the extracellular environment and the nucleus. Extracellular signals such as hormones, cytokines, and various growth factors interact with their receptors to activate the small G-proteins of the RAS family. Active RAS acts via adaptor proteins to activate and recruit RAF proteins to the cell membrane where they are activated. Active BRAF signals through MEK to phosphorylate and activates ERK, which, in turn activates downstream transcription factors to induce a range of biochemical processes including cell differentiation, proliferation, growth, and apoptosis [55]. BRAF is the most potent activator of MEK. Active RAS induces conformational changes in RAF that allows its recruitment to the cell membrane, promoting changes in the phosphorylation status and triggering its kinase activity. Unlike BRAF, ARAF and CRAF require an additional phosphorylation in the N-region of their kinase domain for full activation, likely contributing to the predominance of the BRAF isoform in the activation of MEK [54]. BRAF phosphorylates and activates MEK1/2, which initiate a kinase cascade that acts through ERK1/2 to signal for ligand and cell-specific responses. MEK1/2 contain proline-rich segments in the carboxy-terminal domains that are required for MEK activation by RAF, supported by the observation that deletion of the proline insert from MEK-1 impairs its activation by RAF in transfected cells [56]. RAF/MEK coupling is required for the downstream phosphorylation of ERK1/2. Regulation of this pathway is crucial for the maintenance of homeostasis in response to extracellular signaling. It has been shown that upregulation of this pathway can induce an arrest of the cell cycle whereas hyperactivation of the pathway can initiate tumorigenesis [57].

BRAF- The Oncogene

BRAF is a key molecule of the MEK/ERK proliferative signaling cascade and so an activating mutation in *braf* gives tumors an unrestrained proliferative advantage. The *braf* gene is mutated in a wide variety of human cancers with more than 40 different mutations identified till date. 90% of those are a missense mutation of a single base from Thymine to Adenine at position 1,799 located on Exon 15. This result is a change at residue 600 where Glutamate is substituted by Valine (BRAF V600E). BRAF V600E can gain 500-fold increased activation, causing constitutive activation of signaling through MEK/ERK signaling in tumor cells independent of RAS or any extracellular stimuli.

The initiation of constitutive activation of BRAF can be appreciated only in the context of its structure. Since BRAF is a highly regulated signal transduction kinase, it first binds to RAS-GTP to become an active enzyme. Subsequent activation occurs in two phases- relieving CR1 autoinhibition and CR3 domain activation. The CR3 domain is inhibited by 2 mechanisms, autoinhibition of its own regulatory Ras GTP-binding CR1 domain and lack of post-translational phosphorylation of key serine and tyrosine residues in the CR2 hinge region. During BRAF activation the CR1 domain first binds the RAS-GTP effector domain to the CR1 RAS-binding

domain (RBD) to release the kinase CR3 domain. The CR1-Ras interaction is further strengthened through the binding of the cycteine –rich subdomain (CRD) of CR1 to Ras. Unlike ARAF and CRAF, BRAF is constitutively phosphorylated on CR2 S445. This allows the negatively charged phosphoserine to immediately repel CR1 through stearic and electrostatic interactions once the regulatory domain is unbound freeing the CR3 kinase domain to interact with substrate proteins. Following the autoinhibitory CR1 regulatory domain release, BRAF's CR3 kinase domain has to change to its ATP- binding active conformation before it can catalyze protein phosphorylation. In the inactive conformation, F595 of the DFG motif blocks the hydrophobic Adenine binding pocket while activation loop residues form hydrophobic interactions with the P-loop, stopping ATP from accessing its binding site.

When the activation loop is phosphorylated, the negative charge of the phosphate is unstable in the hydrophobic environment of the P-loop. As a result, the activation loop changes conformation, stretching out across the C-lobe of the kinase domain. In this process, it forms stabilizing β-sheet interactions with the β6 strand. Meanwhile, the phosphorylated residue approaches K507, forming a stabilizing salt bridge to lock the activation loop into place. The DFG motif changes conformation with the activation loop, causing F595 to move out of the adenine nucleotide-binding site and into a hydrophobic pocket bordered by the αC and αE helices. Together, DFG and activation loop movement upon phosphorylation open the ATP binding site. Since all other substrate-binding and catalytic domains are already in place, phosphorylation of the activation loop alone activates B-Raf's kinase domain through a chain reaction that essentially removes a lid from an otherwise-prepared active site.

Thus, all that is required for BRAF to transition to an active state is a change in position of the DFG motif/ activation segment [58]. Phosphorylation of the activation segment results in the destabilization of the hydrophobic interactions between the activation segment and the P-loop, resulting in a flip of the DFG segment to its active state, aligning the ATP and peptide recognition segments, and allowing access to the catalytic cleft. The BRAF V600E mutation replaces V600 valine, a medium sized hydrophobic side chain with E600 glutamine, a larger, charged side chain. V600 is located in the activation segment close to the DFG motif and so the V600E mutation disrupts the hydrophobic interaction, destabilizing the conformation that maintains the inactive orientation of the DFG motif to its active orientation thereby rendering BRAF V600E to a constitutively active state. [59].

BRAF mutation in Melanoma

The RAS/RAF/MEK/ERK pathway is most frequently mutated in melanoma, with RAS mutations predominantly of the *nras* isoform, found in 15-30% and *braf* mutations found upto 70% melanomas. Mutations in BRAF can range from V600 E/K/D/G/R but among them BRAF V600E is the most frequent with >90% melanomas exhibiting this mutation. The second most common mutation is BRAFV600K substituting lysine for valine, that represents 5-6 % (GTG > AAG), followed by BRAFV600R (GTG > AGG), an infrequent two-nucleotide variation of the predominant mutation, BRAF V600 ′E2′ (GTG > GAA), and BRAF V600D (GTG > GAT).

BRAF V600E mutation in melanoma cells cause activation of ERK1/2 without the need for signaling from the extracellular matrix (ECM). As a result CYCLIN D1 is upregulated in the absence of extracellular signals, P27 the CDK inhibitor is downregulated both transcriptionally as well as by upregulated proteasomal degradation which require BRAF and CYCLIND1. Thus BRAF mutation subverts the adhesion and growth factor requirements for ERK1/2 signaling in melanoma cells, thereby allowing upregulated progression through the G1/S checkpoint.

A major advance of the past few years has been the discovery that RAF kinases can homo- and heterodimerize [60, 61], the structure of an active RAF kinase is that of a side-to-side dimer in which only one partner must have catalytic activity [62]. Dimerization is enhanced by Ras [63] and is subject to negative feedback regulation by ERK [64,65]. In addition to causing constitutive activation of BRAF the V600E mutation also renders the protein insensitive to the negative feedback mechanisms.

BRAFV600E has been implicated in different mechanisms of melanoma progression, and principally the activation of the downstream MEK/ERK pathway, evasion of senescence and apoptosis, unchecked replicative potential, angiogenesis (through MEK-dependent activation of HIF-1α and VEGF), tissue invasion and metastasis (via upregulation of several proteins involved in migration, integrin signaling, cell contractility, tumor- and microenvironment-derived interleukin-8), as well as the evasion of immune response [66]. Given the central role of the BRAF signaling pathway in melanoma targeting this pathways with small molecule inhibitors seem like a logical treatment strategy to follow.

Wnt signaling in normal melanocytes

Wnts are a family of 19 secreted lipid-modified, cysteine-rich signaling glycoproteins that are highly conserved from Drosophila to humans. They play key roles in regulating diverse cellular processes ranging from embryonic development including cell proliferation, survival, migration and polarity, adult homeostasis, specification of cell fate and self –renewal in stem cells as well as tumor progression [67,68,69]. Three Wnt signaling pathways have been characterized to date: Canonical Wnt/beta-catenin pathway, Noncanonical Wnt/Calcium pathway, and Noncanonical planar cell polarity (PCP) pathway [70, 71]. The canonical Wnt pathway regulates transcription of a wide spectrum of target genes, while the noncanonical Wnt/calcium pathway controls calcium levels inside the cell and the noncanonical PCP pathway regulates cytoskeleton rearrangement.

Canonical Wnt signaling

β-catenin stabilization upon binding of Wnt1 or Wnt8 to their cognate receptors is the signature of 'canonical' Wnt signaling. In absence of the Wnt ligand, β-catenin level is restricted in the cytoplasm by a multiprotein "destruction complex" composed of glycogen synthase kinase 3beta (GSK3β), adenomatous polyposis coli (APC), Axin, protein phosphatase 2A and β-TrCP. GSK3β phosphorylates β-Catenin at specific regulatory sites at its N-terminus, which leads to its recognition by the ubiquitin ligase Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase β-TrCP, followed by ubiquitination and subsequent proteosomal degradation of the protein.

When the appropriate Wnt binds to its appropriate frizzled receptor, the G-proteins Gαo and Gαq are activated which is thought to activate the cytoplasmic phosphoprotein dishevelled (DVL), which then becomes recruited to the cell membrane. Dsh becomes hyperphosphorylated via multiple kinases such as casein kinase-1 and -2 (CK1/2) [72] and displaces and inhibits GSK3β phosphorylation of β-catenin, without affecting the enzymatic activity of GSK3-β. DVL also blocks Axin to the plasma membrane either by direct interaction or through change in Axin phosphorylation [73]. Activated DVL can also inactivate GSK3-β by interacting with GBP/Frat1, which binds to DVL via its N-terminal PDZ domain and to GSK3-β via its c-terminus [74]. This results in recruitment of the destruction complex, clustering, and subsequent endocytosis of activated receptor complexes into "Wnt-signalosomes" [75]. This complex is then trafficked inside multivesicular bodies (MVBs) in an Endosomal Sorting Required for Transport (ESCRT)-dependent manner [76–78]. The sequestration of GSK3 and Axin inside MVBs is required for Wnt signaling [76,79] such that newly synthesized β-Catenin is can accumulate in the cytoplasm and subsequently translocate to the nucleus, to participate in transcriptional regulation via TCF/ LEF S, lymphoid specific DNA-binding proteins. LEF provide the DNA binding moiety [80] and induce a sharp bend in the DNA helix [81,82], but have no transactivation domains in the absence of β-catenin. During Wnt signaling and when interacting with TCFs, β-catenin provides two transcriptional activation domains located in the C and N termini [83,84].

Many putative targets of the TCF/β -catenin transcriptional complex have been identified both in development like MITF-M, Sox10 and in tumorigenesis like c-Myc, cyclin D1 and PPARδ [85]. Other targets of this pathway include the matrix metalloproteinase, matrilysin [86] and the transcription factors AP-1, c-jun and fra1 [87].

Wnt signaling in melanocyte development

Melanocytes arise from the neural crest, which are initially found at the lateral edge of the neural plate. Wnt family of proteins is involved induction and migration, proliferation and differentiation of the neural crest. Neural crest cells develop into melanoblasts, containing immature melanosomes, which lack functional tyrosinase, the enzyme critical for melanin production. Melanoblasts undergo massive proliferation, and migrate beneath the ectoderm along a dorso-lateral surface. Wnt signaling, specifically Wnt1 and Wnt3a regulate these aspects of early melanocytic development [88]. Additionally it has also been shown that the canonical Wnt signaling is critical for both proliferation of melanocytes, as well as differentiation. Wnt8 has been shown to play a role in pigment cell specification [89].

Various Wnts act in synergy to regulate the expression of well-known melanocyte markers like the micropthalmia tracsription factor, MITF, and the SRY (sex determining region Y)-box 10 transcription factor, SOX10. Wnt signaling is responsible for the initial expression of SOX10 during neural crest induction [90], and later directing the fate of neural crest cells to become pigment cells, via the regulation of dopachrome tautomerase (DCT) another important regulator of pigmentation [91] and MITF [92]. The MITF promoter has a functional LEF-1 binding site, making it a prime Wnt/β-catenin signaling target [93]. MITF controls both the proliferation and survival of melanocytes, as well as their differentiation.

Wnt signaling in melanomagenesis and progression

The role of canonical Wnt signaling in melanomagenesis has been controversial with current literature reporting contradictory findings. A clinical study examined Wnt ligand expression in malignant melanoma and reported high levels different subtypes of Wnt correlating with the histopathological features of the tumor [94]. Melanomas characterised by small, uniform cells strongly expressed Wnt2, Wnt5a, Wnt7b, and Wnt10b. In contrast, melanomas characterized by large, pleomorphic cells expressed Wnt10b but did not express Wnt2 and had low levels of expression of Wnt5a. Expression of Wnt7b was variable in these melanomas. Microarray data implicated Wnt5a and SAGE data indicated Frizzled7 to be playing an important role in transition of melanoma from vertical growth phase to frank metastasis. In another study Wnt2 expression level was high in melanoma cell lines, and antibodies against Wnt2 inhibited b-catenin signaling and suppressed tumor growth in an in vivo xenograft model by inducing apoptosis. Together these studies support an oncogenic role of Wnt in melanoma. Contradictory to these reports B16 melanoma cells expressing Wnt3a exhibited decreased tumor size and decreased metastasis when implanted into mice [95]. A genome-wide transcriptional profiling

performed in lentiviral-transduced B16F1 cells to determine genes upregulated by the expression of Wnt3a, also showed upregulation of genes associated with melanocyte differentiation like MITF, SOX9 Kit suggesting Wnt3a might be driving melanoma cells toward a more differentiated melanocytic cell fate. Another interesting study shows Wnt signaling in melanoma cells regulates their interaction with the niche by affecting their ability to recruit blood and lymph vessels [96]. Cell culture supernatants (SNs) of Wnt1(+) and Wnt1(-) melanoma were added to endothelial spheroids. SNs of Wnt1(-) melanoma cells, but not those of Wnt1(+) cells, induced lymphatic sprouts. Subsequent tests revealed that Wnt1 negatively regulates lymphangiogenesis by suppressing melanoma-derived VEGF-C expression.

Currently not many studies directly implicate Wnt proteins in tumorigenesis. Instead, in many cases, other downstream signaling members such as b-catenin, axin and APC have been shown to be directly involved in tumorigenesis. The role of b-catenin signaling in melanoma development is intriguing given that it too has demonstrated dual roles in both promoting and preventing melanoma progression. Supporting an activating role of b-catenin signaling in melanomagenesis, one study reported that constitutive activation of Wnt/b-catenin signaling enhances cell growth of murine melanoma cells in vitro [96]. Genetic studies in transgenic mice have implicated this pathway as a promoter of melanoma development. Another group has shown that co-expression of stabilized nuclear b-catenin with oncogenic NRas promotes melanoma formation in vivo and increased immortalization of melanocytes in vitro [97]. In the same vein, a conditional mouse model of melanoma, based on melanocyte-specific Pten loss and the BRAF V600E activating mutation, revealed a key role of b-catenin in mediating tumor progression and metastasis [98]. In this Pten/Braf mouse model, increasing or decreasing b-catenin levels leads to enhanced or repressed metastasis formation, respectively. Consistent with this, multiple immunohistochemistry studies of human melanomas revealed that many primary melanoma tumors have elevated levels of nuclear b-catenin. However, in contrast to this, in more malignant and metastatic melanomas, nuclear b-catenin levels appear to decrease with the suppression reportedly brought about by oncogenic BRAF V600E [95,99,100,101]. Supporting these findings down-regulation of Wnt/b-catenin signaling in patient tumors has been associated with decreased patient survival. Overall these studies suggest that the consequences of Wnt/b-catenin signaling activation are complex and likely dependent on the biological context.

Temporal activation of Wnt signaling is perhaps crucial for melanoma development and progression. Just as in development, where a temporally inappropriate activation of Wnts can lead to various embryonic defects, so it is on a cellular level in melanoma. β -catenin expression is perhaps an early event, and metastatic cells down regulate expression of this protein prior to invasion, and escaping the immune system. Similarly Wnt5a may provide a survival advantage to melanoma cells, though in some cancers it is thought to act as a tumor suppressor. Thus, its early expression may result in suppression of tumorigenesis, whereas if it is expressed at a later stage, it becomes a potent inducer of migration and motility. Overall, Wnt signaling and it effects on melanoma onset and progression are complex, and surely temporal and context dependent. While Wnt/catenin signaling may be required for the initiation of melanoma development or melanocyte transformation, additional research is needed to determine the consequences of down-regulation of this pathway in already established melanoma tumors.

MITF-M – The master regulator in melanocytes and melanoma

MITF –M, the isoform of the Micropthalmia Associated transcription factor expressed in normal melanocytes and melanoma is a lineage commitment transcription factor that plays an essential role in melanoblast propagation in early development as well as in melanoma. MITF-M is the master regulator of melanocyte biology playing a central role in their development differentiation, function and survival. Recently it has been implicated as a "lineage survival oncogene" for its central role in melanoma development [102].

MITF is expressed in pigmented cells including melanocytes, retinal pigment epithelium cells, as well as in certain non-pigmented cell lineages including osteoclasts and mast cells [103]. There are at least nine different alternatively spliced transcript variants of MITF that exhibit tissue-specific expression patterns. Isoform M (MITF-M) is exclusively expressed in melanocytes and melanoma [104]. In addition to alternative splicing, MITF transcript variants are also derived from different MITF promoters. In melanocytes the M promoter drives MITF and restricted promoter activation brings about cell type specific expression of the protein. MITF belongs to the family of basic helix-loop-helix leucine-zipper microphthalmia-related transcription (MiT) factors which, form dimers and bind to DNA recognition sequences called E-boxes, with consensus sequence of CA[T/C]GTG.

Heterozygous mutation of MITF has been implicated Waardenburg syndrome Type IIA, an autosomal dominant condition in humans [105]. Mutation in the MITF genes results in an abnormally truncated protein, which disrupts formation of MITF dimers and result in insufficient development of melanocytes. Shortage of melanocytes result in some clinical features of Waardenburg syndrome like congenital white forelock and sensoneural deafness.

Another congenital disorder resulting from mutation in MITF is Tietz syndrome, which is characterized by deafness and leucism [106]. The deletion or single base pair mutation makes the new MITF protein unable to bind to DNA thereby altering melanin production and melanocyte development. Reduced melanocyte numbers lead to hearing loss and decreased melanin production account for light skin and hair color associated with Teitz syndrome.

Melanocyte development and MITF

MITF-M is the earliest reported marker of commitment to a melanocytic lineage and it regulates several cellular processes like differentiation, proliferation, migration, invasion and senescence. Hence MITF-M is rightfully termed as the 'master regulator' of melanocyte biology [107]. It also mediates differentiation effects of the α-melanocyte-stimulating hormone (α-MSH) regulating the transcription of enzymes essential for melanin production in melanocytes like DCT (dopachrome tautomease) and TYR (Tyrosinase). As a result of its direct and critical role in melanin synthesis, mutations in the MITF gene or defects in MITF-M metabolism leads to syndromes with pigmentation defects.

Given its the central role in melanocyte biology, at different stages of melanocytic development, MITF-M activity and expression levels are tightly regulated. The MITF-M promoter has binding

sites for multiple transcription factors like lymphoid enhancer-binding factor 1 (LEF1/TCF), sex-determining region Y-box 10 (SOX10), paired box gene 3 (PAX3) and cAMP-responsive element binding protein (CREB), one cut domain 2 (ONECUT-2), and the mitogen-activated protein kinase (MAPK) [108]. Essentially a variety of signals converge to regulate MITF expression and activity, which in turn determine melanocyte fate. In mice, complete absence of MITF-M leads to characteristics such as white fur, deafness, and small eyes (microphthalmia) due to absence of melanocytes, rather than a defect in melanin synthesis within viable melanocytes. Conversely MITF-M overexpression is associated with melanomagenesis and progression.

MITF in melanoma

Recent evidence from integrated genomic studies show MITF-M amplified in 5-20% of human melanomas [109]. This MITF amplification was associated with a decreased five-year survival of the patients [109]. MITF-M was also shown to control proliferation and metastasis of melanoma cells [109,110,111]. Since MITF-M plays such a decisive role in both differentiation and survival of the melanocytic lineage [112,113,114] success of this lineage depends crucially on proliferative and survival signals that converge on MITF-M. Functional assays demonstrated that MITF-M can cooperate with activated BRAF V600E as well as the dysregulated p16-CDK4-Rb pathways to transform immortalized human melanocytes. Therefore by directing melanocytic lineage survival during development as well as by promoting melanomagenesis as a result of genetic alterations, MITF-M seems to function as a "lineage-survival" or "lineage addiction" oncogene [115].

MITF-M has been reported to have a paradoxical role in melanomagenesis, conferring either pro or anti survival effects. In-vivo this can be a result of different niche dependent context as well as different genetic backgrounds of individual cells. For example even though 20% of metastatic melanoma cells show amplification of MITF-M, but the effect of increased copy number can be dampened by transcriptional and post-translational regulation [116]. Oncogenic BRAF stimulates BRN2 expression exclusively in melanoma to acquire control of MITF-M promoter to regulate MITF-M expression and BRN2 depletion negates the ability of BRAF to activate MITF-M in human melanoma cells [117].

For BRAF mutant/MITF-M-positive melanoma cells, a BRAF/BRN2 rheostat mechanism [116] has been proposed to control MITF-M basal expression levels. Presence of oncogenic BRAF induces strong MAPK pathway activation, leading to high levels of expression of BRN2 that is required for BRAF regulated control of MITF-M expression. However BRAF maintains MITF-M at a certain optimal expression level to ensure efficient survival and proliferation but prevents upregulation of MITF to the level where it triggers a cell cycle exit and differentiation via induction of cellular senescence and melanin production as well as downregulation to the extent where it triggers apoptosis. This model also underscores the theory that oncogenic BRAF co-operates with appropriate MITF-M levels to drive melanoma initiation. This cell population is therefore sensitive to BRAF inhibitors more than cells where the oncogenic BRAF pathway is not active and does not regulate MITF-M expression. One such population is the wild type BRAF expressing WNT positive cells that are resistant to MAPK pathway inhibitors partly due to the fact that MITF expression is disconnected from oncogenic BRAF. Taken together the role

of MITF-M in melanoma genesis is 'two-faced' and a tight regulation of expression and activity determines cell fate between normal melanocytes and melanoma.

Although it is tempting to theorize targeting MITF-M directly would yield positive therapeutic outcomes in malignant melanoma, practically it is difficult to "drug", since it lacks a ligand binding pocket and measurable catalytic activity. However it is logical to hypothesize that targeting "druggable" components signaling upstream of MITF-M could potentially affect it in a desired way to treat melanoma. This dissertation focuses on disrupting MITF-M expression by targeting two different such regulatory pathways namely BRAF/MEK/ERK as well as the canonical Wnt signaling pathway using a natural and synthetic small molecule to bring about an anti-cancer effect in melanoma.

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CHAPTER 1

Indole-3-carbinol directly inhibits oncogenic BRAF activity to disrupt MITF-M expression and arrests melanoma cell proliferation

ABSTRACT

Indole-3-carbinol (I3C), a natural phytochemical derived from the hydrolysis of glucobrassicin produced in cruciferous vegetables, such as broccoli and Brussels sprouts strongly inhibited proliferation and down regulated protein levels of the *melanocyte master regulator Micropthalmia Associated Transcription Factor (MITF-M) in oncogenic BRAF-V600E expressing melanoma cells maintained in cell culture and in vivo in tumor xenografted athymic nude mice. In contrast, wild type BRAF-expressing melanoma cells remained relatively insensitive to I3C anti-proliferative signaling and MITF-M expression and protein levels did not show any decrease.* Importantly, normal human epidermal melanocytes were unaffected by I3C treatment. *Western blots revealed a corresponding decrease in MITF-M target proteins that are involved in regulating the cell cycle like CyclinD1, CDK 2,4,6 in I3C sensitive cells whereas in wild type BRAF expressing I3C insensitive cells, no such effect on cell cycle regulators were observed. This inhibition of cell cycle regulators by I3C produced a G1 cell cycle arrest, which was determined by flow cytometric analysis on cell lines treated with I3C. In BRAF-V600E-expressing melanoma cells, I3C treatment inhibited phosphorylation of MEK and Erk/MAPK, which are down stream effectors of BRAF. The I3C anti-proliferative arrest was concomitant with the down-regulation of MITF-M transcripts and promoter activity, loss of endogenous BRN-2 binding to the MITF-M promoter, and was strongly attenuated by expression of exogenous MITF-M. In silico* modeling using known crystallographic structures of BRAF proteins predicted a thermodynamically stable I3C binding site within the HRD motif in the BRAF-V600E catalytic loop, whereas only low affinity interactions outside the catalytic domain were predicted with the wild type BRAF. *In vitro* kinase assays using immunoprecipitated BRAF-V600E and wild type BRAF demonstrated that I3C directly inhibited the enzymatic activity of only the oncogenic BRAF but not of the wild type protein. Compared to the effects of each compound alone, combinations of I3C and the clinically used BRAF-V600E inhibitor, Vemurafenib cooperatively inhibited melanoma cell proliferation and reduced MITF-M levels in BRAF-V600E expressing melanoma cells. Taken together, our results identify oncogenic BRAF-V600E as a new cellular target of I3C that implicate this natural indolecarbinol compound as a potential candidate for novel single or combination therapies for human melanoma.

INTRODUCTION

Human melanoma, a melanocytic neoplasm of the skin, accounts for only 5% of all diagnosed skin cancers but 75% of all skin cancer related deaths [1]. The high mortality rate is due to its high propensity to metastasize as well as refractoriness to existing therapies. Advances in high throughput sequencing technology have aided in genome profiling of these cells and provided insights into tumor heterogeneity, molecular features as well as the genetic diversity of melanomas. The identification of critical somatic events, complemented by functional studies has helped determine the key signaling pathways and processes affected by these changes. This understanding has led to classification of these tumors based on some common driver mutations, which not only determine the cellular phenotype but also the therapeutic options for patients.

Well-established driver mutations of melanoma include activating mutations in the proto-oncogenes BRAF and NRAS and inactivating mutations in the tumor suppressor proteins such as PTEN, TP53 and p16INK4a [2]. More than 60% of melanoma patients express a point mutation within a hot spot at position 600 on the BRAF gene. Approximately 90% of these mutations result in a T1799A transversion that substitutes Valine for Glutamate at residue 600 in the kinase domain of the protein. Other BRAF V600 mutations include V600K/D/R all of which lead to hyperactivation of downstream MEK/ERK signaling. All the BRAF V600 mutations cause >200-500 fold increase in activity of this Ser/Thr protein kinase and result in the constitutive activation of signaling through the BRAF/MEK/ERK pro-proliferative pathway [3].

Interestingly in human melanomas, the neuronal specific transcription factor BRN2 (N-Oct-3) is reactivated to be regulated by ERK and BRN2 in turn regulates the transcription of the melanocyte master regulator MITF-M. This allows the oncogenic BRAF to take charge of MITF-M expression and thereby melanoma biology [4]. *Elevated MITF-M levels have been shown to correlate with decreased overall patient survival [5]. Recent reports indicate MITF-M to be a 'lineage survival' oncogene that is required for both tissue-specific tumorigenesis and progression [6]. Furthermore, one mechanism of acquired resistance to BRAF inhibitors involves the amplification and restoration of transcriptional activity of MITF-M [7]. Hence, the levels of MITF-M can be considered a critical factor that determines the efficacy of a given melanoma targeted therapy and probability of relapse of the disease.*

Indole-3-carbinol (I3C), a natural indolecarbinol compound derived from hydrolysis of glucobrassicin produced in cruciferous vegetables of the *Brassica* genus such as *broccoli, Brussels sprouts, and cauliflower*, is a promising anti-cancer molecule because of its anti-proliferative effects in a wide range of human cancers with negligible toxicity and minimal side effects [8-11]. I3C activates several distinct and complementary anti-proliferative signaling cascades in human cancer cells [12-16], and is *currently in clinical trials for treatment and prevention of breast and prostrate cancer, respectively [17]*. We originally established in different subtypes of human breast cancer cells that I3C induces its anti-proliferative response by the direct inhibition of elastase enzymatic activity and subsequent regulation of CD40-directed cell signaling cascades [18-19]. *Thus,* an essential concept that emerged from our studies is that the presence of specific I3C target proteins expressed in human cancer cells mediates the

efficacy by which I3C selectively stimulates distinct anti-proliferative signaling cascades [13-16, 20].

In human melanoma [21] and squamous cell carcinoma [22], I3C treatment has been shown to increase sensitivity to UV induced apoptosis and enhance cytotoxic responses, respectively. Also, ectopic application of I3C directly inhibits skin tumor formation in mouse models [23]. However, relatively little mechanistic information has been uncovered concerning the effects of I3C on skin cancers. We have observed that human melanoma cells with distinct mutational profiles are sensitive to different extents to the anti-proliferative effects of I3C [21], suggesting that the ability of I3C to trigger its anti-cancer signaling is linked to its interaction with specific melanoma target proteins expressed in each cell type. In this regard, the study covered in Chapter 2 of this dissertation demonstrates that I3C directly binds to the NEDD4-1 ubiquitin ligase and induces the stabilization of the wild type PTEN tumor suppressor protein. The enhanced levels of wild type PTEN triggers the loss of activated Akt cell survival signaling; however, this effect is limited to the subset of melanoma cells expressing wild type PTEN [21].

In the present study, we demonstrate that I3C shows significant antiproliferation in melanoma cells in culture and anti-tumor effect in melanoma xenografted tumors in-vivo. However I3C has no such detrimental effect on normal epidermal melanocytes indicating a cancer specific mechanism of action. I3C selectively causes a G1 cell cycle arrest in sensitive melanoma cell lines by downregulating critical cell cycle regulators like Cyclin D1, CDK2, 4, 6, which are in turn regulates MITF-M. I3C strongly disrupts MITF-M expression in sensitive melanoma cell lines by inhibiting oncogenic BRAF signaling. I3C directly inhibits oncogenic BRAF-V600E kinase activity with no corresponding effect on the wild type BRAF protein. This selective interaction accounts for the loss of down stream BRAF-V600E signaling, reduced MITF-M gene expression and elevated sensitivity of oncogenic BRAF expressing melanoma cells to the anti-proliferative effects of I3C. Furthermore, combinations of I3C and Vemurafenib, a clinically employed oncogenic BRAF inhibitor, cooperatively down-regulates MITF-M expression and inhibit melanoma cell proliferation, thereby implicating the potential use of I3C-based compounds in the development of new monotherapeutic or combinational therapies for human melanoma.

MATERIALS AND METHODS

Cell Culture

Melanoma cell lines G-361, SK-MEL-2, SK-MEL-24 and RPMI-7951 were purchased from American Type Culture Collection (ATCC), and were authenticated according to the ATCC guidelines. DM738 melanoma cells were acquired from the tissue culture facility at University of California, Berkeley. The G361 melanoma cells were cultured in Modified McCoy's 5A cell media supplemented with 10% fetal bovine serum (Gemini Bio Products, 2 mM L- glutamine, and 2.5 ml of 10,000 U/ml penicillin/streptomycin mixture (Gibco, Life Technologies). SK-MEL-24 cells were cultured in DMEM with 4.5 g/L glucose supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2.5ml of 10,000 U/ml penicillin/streptomycin mixture in addition to 1X MEM Non-Essential Amino Acid (Gibco, Life Technologies). DM738, RPMI-7951 and SK-MEL-2 melanoma cells were cultured in DMEM containing 4.5 g/L Glucose, 114 mg/L Sodium Pyruvate, and 2 mM L-glutamine, supplemented as described above. The cells were incubated in tissue culture dishes (Nalgene Nunc) at 37°C with controlled humidity and 5% CO2 air content.

Treatment with Indole-3-carbinol, Vemurafenib and U0126

I3C was purchased from (Sigma Aldrich), Vemurafenib from (Adooq Bioscience) and U0126 (Cell Signaling). To study the effect of I3C, cells were treated with or without 200 μM I3C every 24 hours for 72 hours and harvested at 24, 48 and 72 hours for western blot and flow cytometric analysis. Cells were treated with Vemurafenib and U0126 for 48 hours before harvesting them for western blots. For the experiments on the combination of I3C and Vemurafenib treatment, cells were treated with the compounds for 24 hours. I3C, Vemurafenib and U0126 were dissolved in 99.9% HPLC grade DMSO (Sigma Aldrich) and the final dilution was performed in the media aliquots used for treatment.

MTT proliferation assay

Melanoma cell lines were seeded on a 48well plate in triplicates and upon 80-90% confluency were either treated with different concentrations of 1-benzyl I3C, I3C or Vemurafenib for 48hours, combinations of 1-benzyl I3C and Vemurafenib or DMSO for 24 hours. Subsequently inhibition of proliferation was assessed using the Cell counting Kit -8 (Dojindo) as per the protocol in the user's manual. Briefly, 50μl of the CCK-8 solution was added to each well along with 450 μl media and incubated for 2-3 hours. The absorbance was read at 450nm and % inhibition was calculated for each condition standardizing DMSO to zero using the formula: [(100 – Value of treatment/ Value of DMSO treated control) X100]

Flow cytometry analysis of DNA content

Melanoma cells were either treated with the indicated concentrations of I3C in triplicates cell cultures for 48 hours, or treated with 200 μM I3C in triplicate cultures for a 72 hour time course. The DNA content of propidium iodide stained nuclei from harvested cells were determined by flow cytometry. Briefly, cells were hypotonically lysed in 300 mL of DNA staining solution (0.5 mg/mL propidium iodide, 0.1 % sodium citrate, and 0.05 % Triton-X 100). Emitted fluorescence from the nuclei of wavelengths more than 585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Ten thousand nuclei were analyzed from each sample at a rate of 300-500 nuclei/second. The percentage of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

Western blot Analysis

Western Blot analyses of samples electrophoretically fractionated on 12% acrylamide gels were carried out as previously described [19, 20]. ECL Lightening reagents were used to visualize the primary antibody bound protein bands in nitrocellulose membranes and the results captured on ECL Autoradiography Film (GE Healthcare). The western blots employed the following primary antibodies: mouse anti-MITF-M (Thermo- scientific), mouse anti-HSP 90 (BD Biosciences), mouse anti-CDK2, rabbit anti-CDK4, mouse anti-BRAF, rabbit anti-ERK-p, rabbit anti-ERK1/2 (Santa Cruz), rabbit anti-P21, mouse anti-Cyclin D1, rabbit anti-MEK-p, rabbit anti-MEK1/2 (Cell signaling).

RT-PCR analysis

Total RNA was extracted from harvested cells using the RNeasy extraction kit (Qiagen) for mammalian cells and spectrophotometrically quantified by absorbance at 260 nm. Reverse transcription (RT) reactions were carried out using RT-MMLV reverse transcriptase (Invitrogen) and the cDNA was used for PCR reactions using 10 pM of the following primers: MITF-M forward 5' CCG TCT CTC ACT GGA TTG GT 3', MITF-M Reverse 5' TAC TTG GTG GGG TTT TCG AG 3' GAPDH forward, TGAACGGGAAGCTCACTGG and reverse, TCCACCACCCTGTTGCTGTA. PCR conditions were as follows: 30s at 94 ^{o}C, 30s at 55 ^{o}C and 30s at 72 ^{o}C for 28 cycles. PCR products were electrophoretically fractionated in 1.5 % agarose gels containing 0.01% Gel Red (Biotium) for DNA staining along with 1kb plus DNA ladder and further visualized by a UV transilluminator.

Tumor Xenografts and Immunofluorescence

Approximately 1 million G361 melanoma cells in a total volume of 0.1 ml of Matrigel (Corning Life Sciences) were injected subcutaneously into each lateral flank of NIH III athymic nude mice. The resulting tumors were allowed to grow to a mean starting volume of 146 ± 10 mm^3. The animals were then randomized into two groups of 5 animals each ensuring that the average tumor volume in each group was comparable. The vehicle control group was treated with DMSO and the I3C treated group was injected daily with 200mg/kg body weight of I3C. The resulting tumor volumes were measured every other day for four weeks using calipers. The tumor volumes were calculated using the standard formula: ($Width^2$ X Length)/2, and changes in tumor volumes were calculated using the formula: 100+{(Tf-Ti)/ Ti X100}, where Tf is the final mean tumor volume and Ti is the initial mean tumor volume. The data represents mean ± SEM (*, $P < 0.01$) for 5 mice per group, each with two tumors, one in each flank. At terminal sacrifice, tumor xenografts were harvested, and a portion of each tumor was fixed in 4% para-formaldehyde for 1 hour at room temperature, followed by a PBS wash and subsequently immersed in 3% sucrose overnight at 4°C. The resulting tissues were embedded in OCT and 10 μm thin sections were taken for immunofluorescence studies.

Luciferase reporter assay.

G361 Melanoma cells were cultured to approximately 80% confluency in 6 well adherent culture plates (Nalgene, Nunc) in triplicates for each experimental condition for 24 hours. The cells were then transiently transfected with either a wild-type pGL2-pMITF -333/+120- luciferase reporter plasmid or a similar plasmid with the BRN2 binding site mutated at - 48, pGL2-pMITF-333/+120-ΔBRN2-luciferase; (ΔBRN2) generated using the following primers:

BRN2 Forward, 5'-TACATGCATAACTAGCGAGCTTAGGTTATTATAAGC-3' BRN2 Reverse, 5'- GCTTATATTAACCTAAGCTCGCTAGTTATGCATGTA-3'. PCR conditions used were as follows: 30s hotstart at 95°C, 30s at 95°C, 1min at 55°C, 8 min at 68°C for 16 cycles. 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). SuperFect Transfection Reagent (Qiagen) was used for the transfections as per instructions in the user manual. The next day the cells were either treated with 200μM I3C or an equal volume of the DMSO vehicle control for both wild-type plasmid transfected as well as mutant plasmid transfected cells. 24 hours later luciferase assays were performed by harvesting cells in 1X Promega Lysis Buffer, allowing it to incubate for 15 minutes at room temperature, followed by 15minutes incubation on ice. The cells were then pelleted by a spin at 15,000 rpm for 1 minute at 4°C. 20μl of supernatant from each sample was combined with 100μl luciferase substrate (Promega) and emitted fluorescence was measured using luminometer, Lumat LB 9507 (EG&G Berthold) and the relative light units (RLU) were recorded. The RLU/μg for each sample was calculated by dividing the data obtained with their specific protein concentration determined by a Bradford assay using 1X BioRad Protein Assay reagent and measuring O.D at 595nm.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed using G361 melanoma cells treated with or without 200 μM I3C for 72 hours. Proteins were cross-linked to DNA by the addition of formaldehyde at 2.5% of the final concentration for 5 minutes at room temperature to cultured cells. Fixation was quenched with glycine for a final concentration of 125 mmol/l for 5 minutes. Harvested cells were sonicated and the chromatin was immunoprecipitated overnight and 15μl of mouse anti-Brn2 antibodies (Santa Cruz Biotechnology) was added to the cell lysates to immunoprecipitated the chromatin-bound Brn2 protein. A fragment of the MITF-M promoter (-170 +120) containing the BRN2 binding site (-53 to -27) was amplified using the primers-

MITF-M Forward: 5'- CGT CAC TTA AAA AGG TAC CTT TAT ATT TAT G-3' MITF-M Reverse: 5' –TGT TTTAGC TAG CAC CAA TCC AGT GAG AGA CGG-3' by cycling 36 times (95^{o}C, 30 s/52^{o}C 30 s/72°C, 30 s) with a 72°C, 10 min extension. The PCR products were electrophoretically fractionated on a 1.5% agarose gel and visualized using a transilluminator.

In vitro BRAF Kinase Assay

BRAF immunoprecipitations were carried out either using G-361 cells treated with DMSO, I3C or Vemurafenib for 72 hours or from untreated G-361 and SK-MEL-2 cells as described previously [43]. BRAF was pulled down from the cell lysates using anti-BRAF antibodies bound to protein G-coupled Sepharose beads, while IgG was used as a negative control. Post-immunoprecipitation supernatants were probed with mouse anti BRAF antibody by western blots to determine pull-down efficiency. Only in the "In-Vitro treated" samples DMSO, I3C and Vemurafenib was added to the immunoprecipitated wild type and mutant BRAF prior to the kinase assay, and reactions were incubated at 30°C for 2 hours. Kinase assay was subsequently performed with the immunoprecipitated wild type or oncogenic BRAF from the pre-treated as well as the "in-vitro treated" samples by incubation with recombinant inactive MEK(K97R) (Millipore) supplemented with ATP/Mg^{+2} in kinase reaction buffer at 30°C for 30mins. The reactions were terminated by adding 4x SDS sample buffer and boiling the mixture at 100°C for 5 min. The samples were then electrophoresed, immunoblotted and probed for relative protein levels of phosphorylated MEK and total MEK to assess the kinase activity of the immunoprecipitated BRAF.

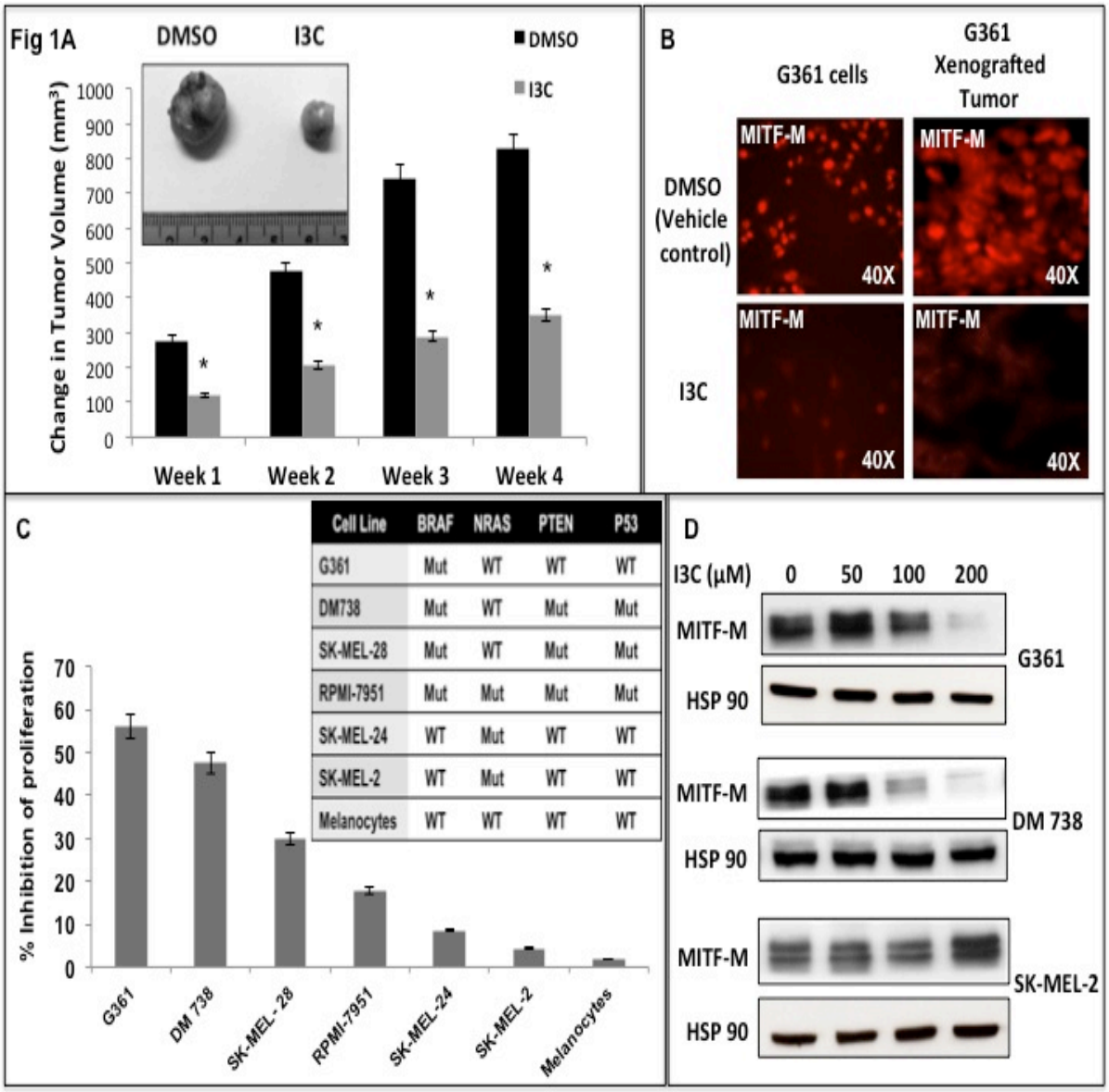


Fig 1A
DMSO
I3C
DMSO
I3C
Change in Tumor Volume (mm³)
1000
900
800
700
600
500
400
300
200
100
0
Week 1
Week 2
Week 3
Week 4
B
G361 cells
G361 Xenografted Tumor
DMSO (Vehicle control)
I3C
MITF-M
40X
C
% Inhibition of proliferation
70
60
50
40
30
20
10
0
G361
DM 738
SK-MEL- 28
RPMI-7951
SK-MEL-24
SK-MEL-2
Melanocytes
Cell Line | BRAF | NRAS | PTEN | P53
G361 | Mut | WT | WT | WT
DM738 | Mut | WT | Mut | Mut
SK-MEL-28 | Mut | WT | Mut | Mut
RPMI-7951 | Mut | Mut | Mut | Mut
SK-MEL-24 | WT | Mut | WT | WT
SK-MEL-2 | WT | Mut | WT | WT
Melanocytes | WT | WT | WT | WT
D
I3C (μM)
0
50
100
200
MITF-M
HSP 90
G361
DM 738
SK-MEL-2

Figure 1.

Effects of I3C on in vivo growth of melanoma tumor xenografts, production of MITF-M, and melanoma cell proliferation.

A. Athymic mice with G-361 cell-derived tumor xenografts were injected subcutaneously with either 200μM I3C or with 200μM DMSO vehicle control, and resulting tumor volumes were calculated as described in the Material and Method section. The data represents mean tumor volume ± SEM for n=5 animals per group, each with two tumors, one in either flank. (*, $p < 0.01$) compared to the control group. The micrograph insert shows tumors harvested at week 4.

B. At terminal sacrifice, tumor sections were analyzed for MITF-M expression by immunofluorescence using primary antibodies to MITF-M (Right panel). Cultured G361cells treated with I3C for 72 hr were similarly probed for MITF-M levels (Left panel).

C. Human melanoma cell lines with distinct genotypes were treated with or without 200μM I3C for 48 hours and the effects on cell proliferation measured using a CCK-8 assay relative to the vehicle control.

D. The levels of MITF-M protein were determined in melanoma cells treated with the indicated concentrations of I3C for 48 hours by western blots.

Fig. 2

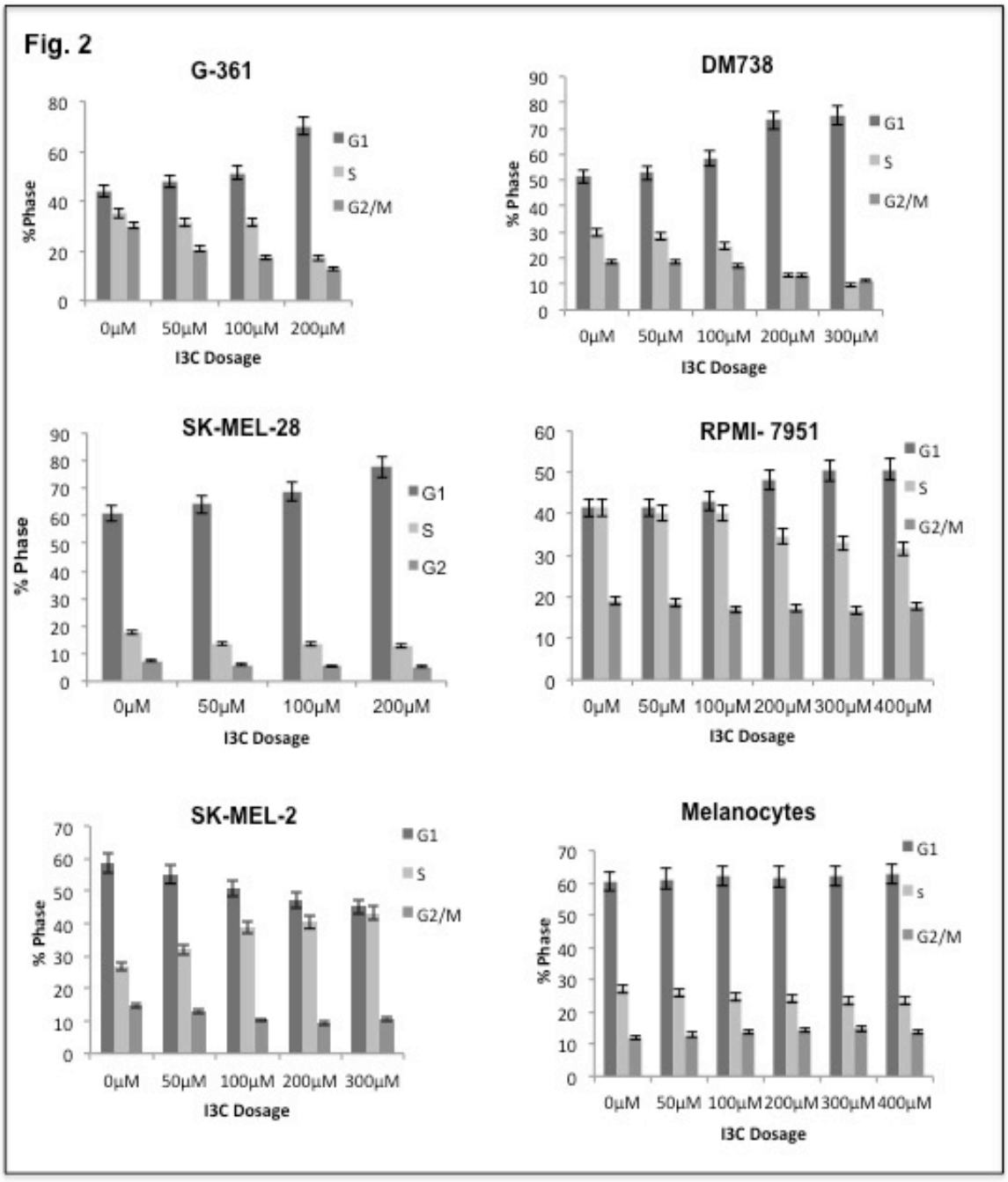

G-361
% Phase
I3C Dosage
0μM
50μM
100μM
200μM
G1
S
G2/M
DM738
300μM
SK-MEL-28
G2
RPMI- 7951
400μM
SK-MEL-2
Melanocytes
s

Figure 2.

Dose dependent cell cycle effects of I3C in G361, DM738 and SK-MEL-2 melanoma cells.

Human melanoma cells expressing either BRAF-V600E (G361, DM738 and SK-MEL28, RPMI-7951) wild type BRAF expressing (SK-MEL-2) and normal epidermal melanocytes were treated with the indicated concentration of I3C and harvested cells were stained with a hypotonic solution containing propidium iodide, and the DNA content of stained nuclei were quantified as described in the supplemental section. The histograms of representative experiments from three independent experiments are shown and the percentage of cells in the population displaying G1, S or G2/M DNA content was quantified. As shown in this figure, I3C induced a significant G1 cell cycle arrest of BRAF-V600E expressing G361 and DM738 cells at the maximal dose of 200 μM I3C. In contrast, wild type BRAF expressing SK- MEL-2 cells remained in a rapidly proliferating state at all tested concentrations of I3C.

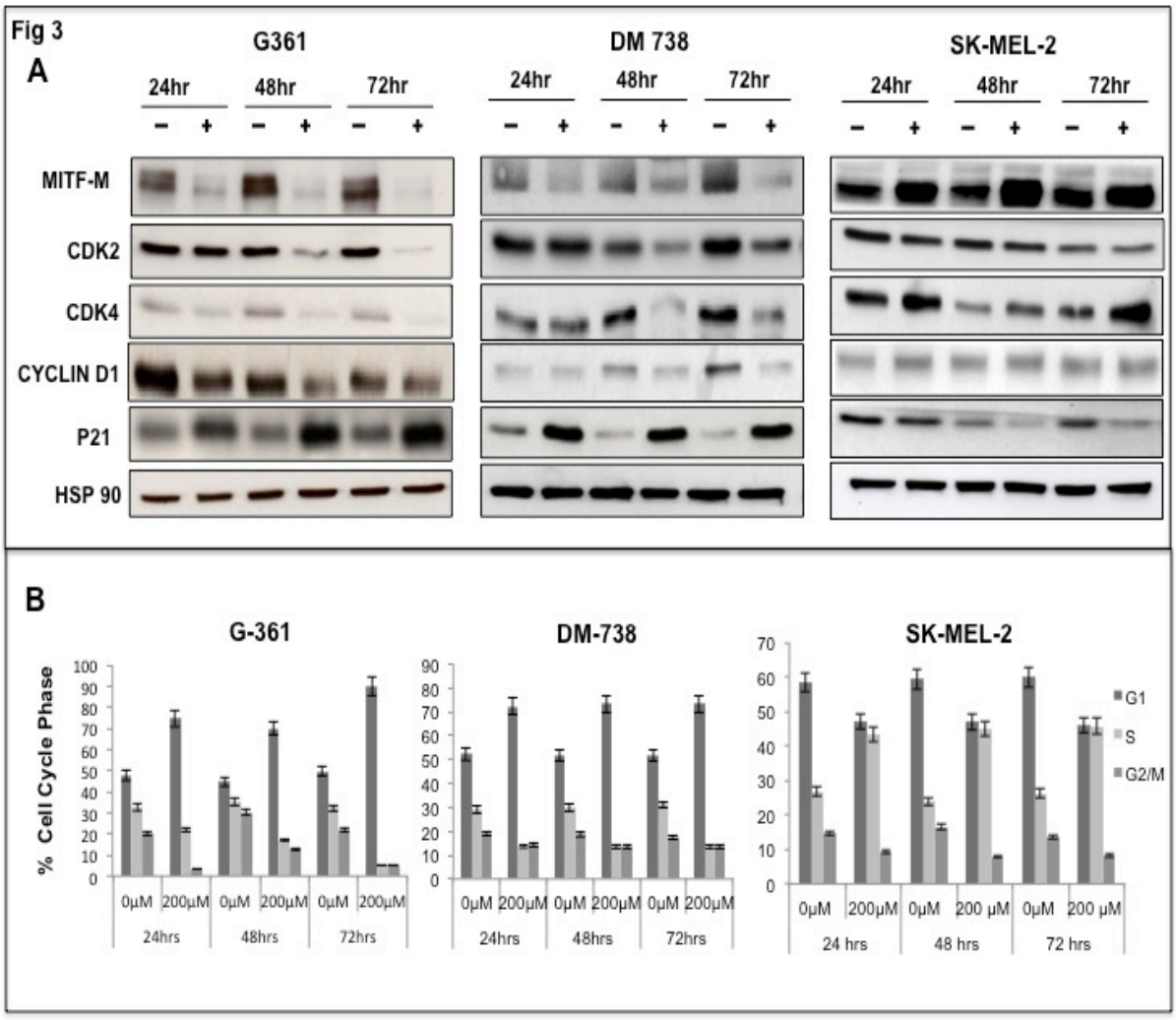


Fig 3
A
G361
DM 738
SK-MEL-2
24hr
48hr
72hr
MITF-M
CDK2
CDK4
CYCLIN D1
P21
HSP 90
B
G-361
DM-738
SK-MEL-2
% Cell Cycle Phase
0μM
200μM
24hrs
48hrs
72hrs
24 hrs
48 hrs
72 hrs
G1
S
G2/M

Figure 3.

Time course of the cell cycle effects of I3C in human melanoma cells expressing BRAF-V600E or wild type BRAF.

Cultured human melanoma cell lines that express either an oncogenic mutant BRAF (G361 or DM738) or wild type BRAF (SK- MEL-2) were treated with or without 200 μM I3C over a 72 hour time course.

A. Total cell lysates were fractionated by SDS-polyacrylamide electrophoresis and the levels MITF-M, and CDK2, CDK4, Cyclin D1, p21 were determined by western blot analysis in comparison to the HSP90 gel loading control. Representative blots from three independent experiments are shown.

B. Cells were stained with a hypotonic solution containing propidium iodide, and the DNA content of stained nuclei were quantified by flow cytometric analysis as described in the methods and materials section. Representative histograms from three independent experiments are shown and the percentage of cells in the overall population displaying G1, S or G2/M DNA content was quantified.

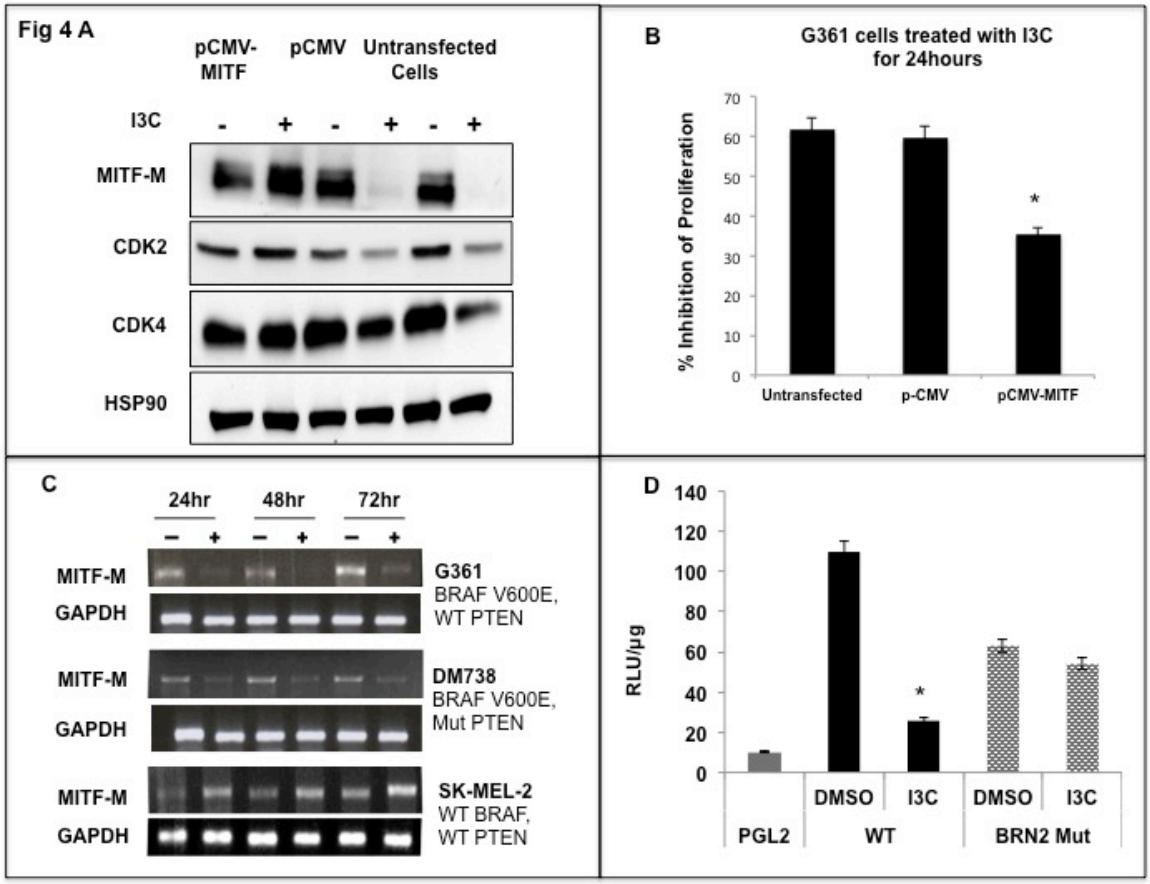


Fig 4 A
pCMV-MITF
pCMV
Untransfected Cells
I3C
- + - + - +
MITF-M
CDK2
CDK4
HSP90
B
G361 cells treated with I3C for 24hours
% Inhibition of Proliferation
70
60
50
40
30
20
10
0
*
Untransfected
p-CMV
pCMV-MITF
C
24hr
48hr
72hr
– + – + – +
MITF-M
GAPDH
G361
BRAF V600E,
WT PTEN
MITF-M
GAPDH
DM738
BRAF V600E,
Mut PTEN
MITF-M
GAPDH
SK-MEL-2
WT BRAF,
WT PTEN
D
RLU/µg
140
120
100
80
60
40
20
0
*
PGL2
DMSO
I3C
DMSO
I3C
WT
BRN2 Mut

Figure 4.

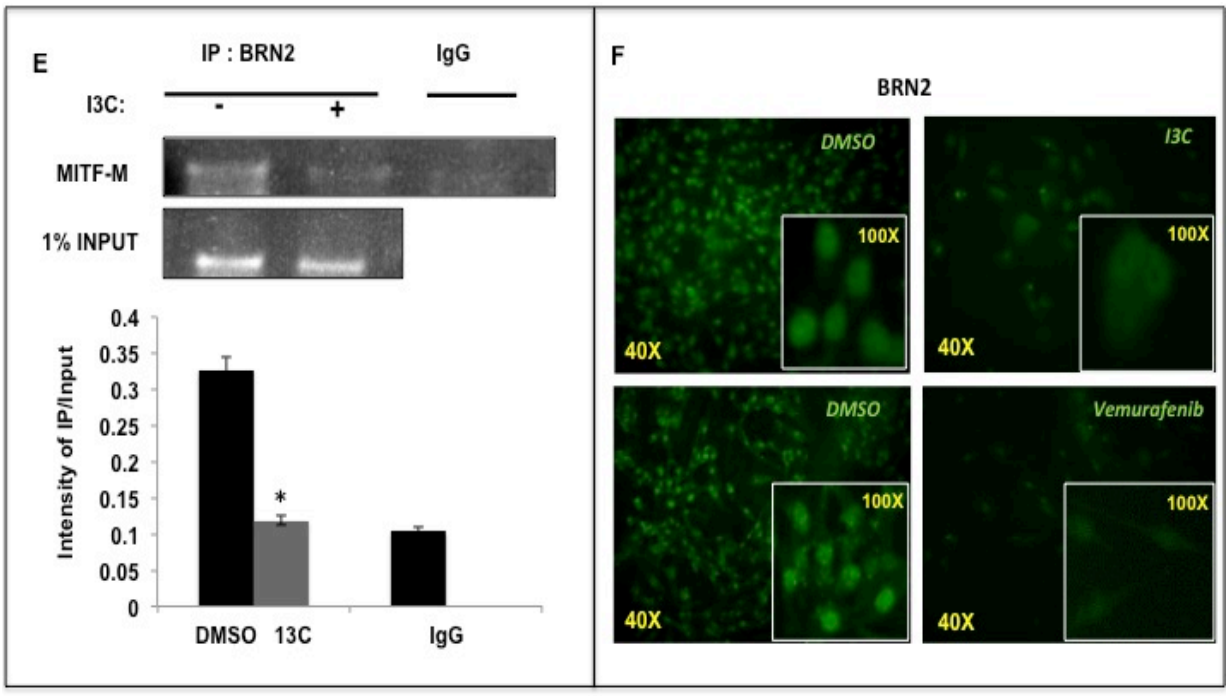
Role of MITF-M in I3C anti-proliferative response and I3C regulation of MITF- M gene expression.

A. G-361 cells were either transfected with pCMV-MITF expression vector, or pCMV empty vector control or left untransfected, and each set of cells were treated with or without 200 μM I3C for a submaximal time of 24 hours. Levels of MITF-M, CDK2, CDK4 and HSP90 protein were determined by western blots (Left panel).

B. Cell proliferation was measured using a CCK-8 assay, and results show the mean of three independent experiments ± SEM (*, $p < 0.01$)

C. MITF-M transcript expression in G-361 cells treated with or without 200 μM I3C was determined by RT-PCR analysis in comparison to the GAPDH control.

D. Cells were transfected with reporter plasmids containing either a wild type MITF-M promoter (WT), a Brn2 consensus site mutant (Brn2 Mut) or the PGL2 empty control vector. Luciferase specific activity was measured in cells treated with or without 200 μM I3C for 24 hours. The bar graph shows the results of three independent experiments in triplicate ± SEM (*, $p < 0.01$).


E
IP : BRN2
IgG
I3C:
-
+
MITF-M
1% INPUT
Intensity of IP/Input
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
DMSO
13C
IgG
F
BRN2
DMSO
100X
40X
I3C
100X
40X
DMSO
100X
40X
Vemurafenib
100X
40X

E. ChIP assay was performed on G361 cells treated with or without 200 μM I3C for 48 hours using BRN2 antibodies (IP:BRN2) or the control IgG with one percent input as the loading control. The bar graphs quantify the densitometry results from three independent experiments ± SEM (*, $p < 0.01$).

F. G-361 cells were treated with or without 200 μM I3C and Vemurafenib for 48 hours and BRN2 localization was examined by immunofluorescence using anti-BRN2 antibodies. The inserts are magnified portions of the larger fields.

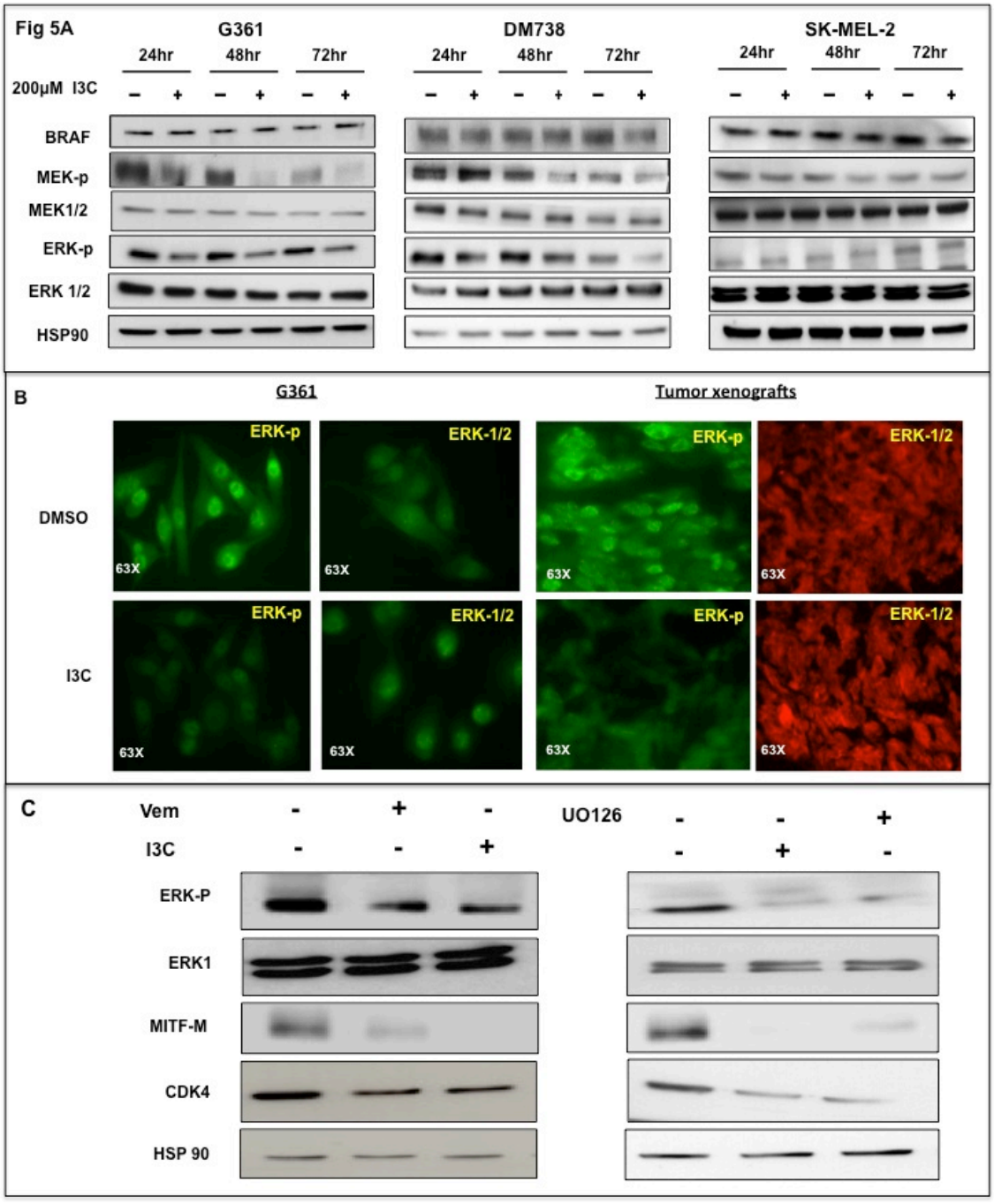


Fig 5A
G361
DM738
SK-MEL-2
24hr
48hr
72hr
200μM I3C
BRAF
MEK-p
MEK1/2
ERK-p
ERK 1/2
HSP90
B
G361
Tumor xenografts
ERK-p
ERK-1/2
DMSO
I3C
63X
C
Vem
I3C
UO126
ERK-P
ERK1
MITF-M
CDK4
HSP 90

Figure 5.

Effect of I3C on BRAF signaling in cells and *in vivo* tumors

A. BRAF-V600E expressing G-361 and DM-738 cells as well as wild type BRAF expressing SK-MEL-2 cells were treated with or without 200 μM I3C for 24, 48 and 72 hours. Western blots were performed on total cell extracts and probed with the indicated antibodies.

B. Tumor xenograft sections from I3C treated and untreated animals were analyzed for ERK-p and total ERK-1 protein by immunofluorescence.

C. G-361 cells were treated with or without 10 μM Vemurafenib BRAF inhibitor, 10 μM U0126 MEK inhibitor or 200 μM I3C for 48 hours. Western blots were probed for the indicated proteins and the results are representative of three independent experiments.

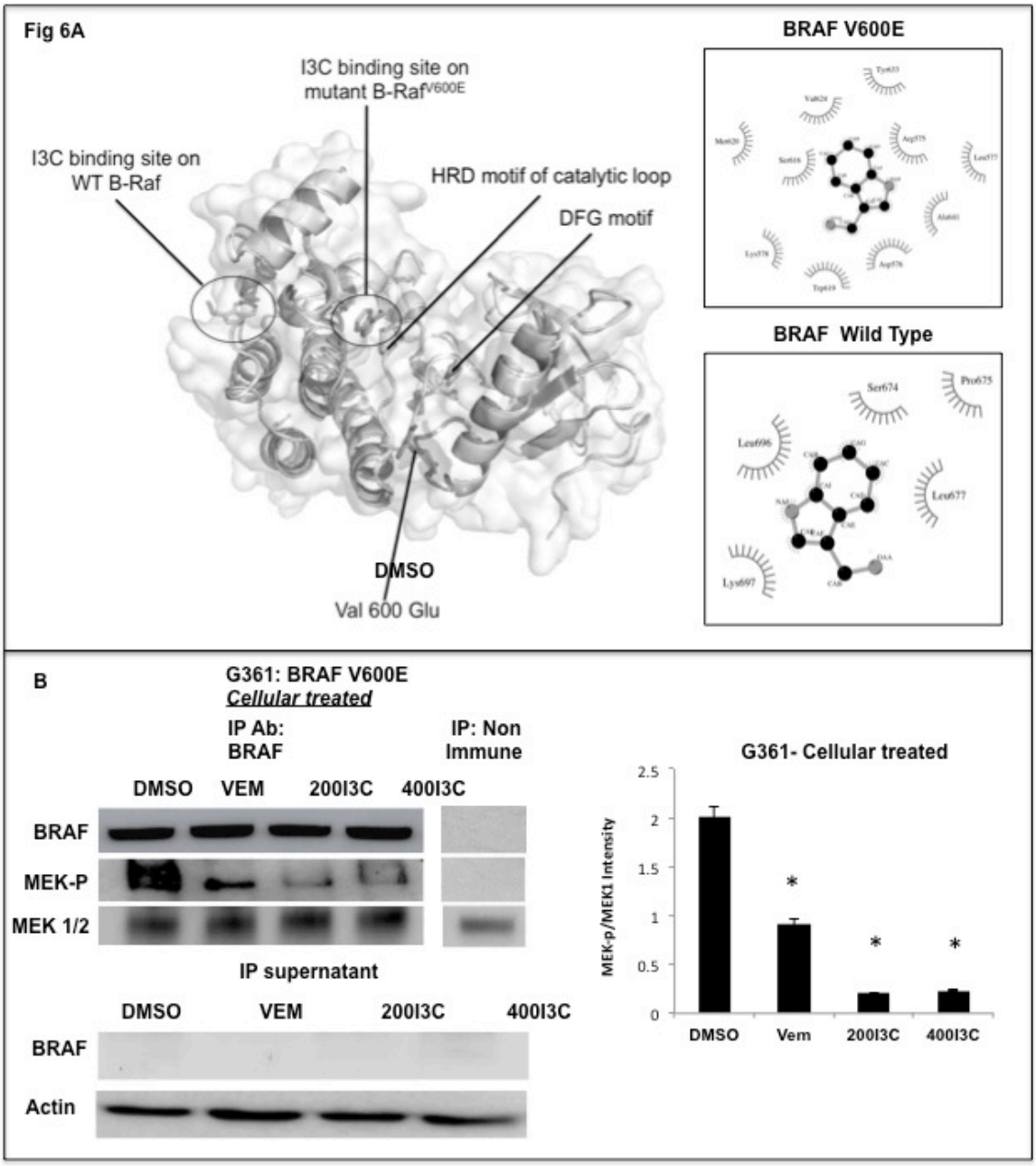


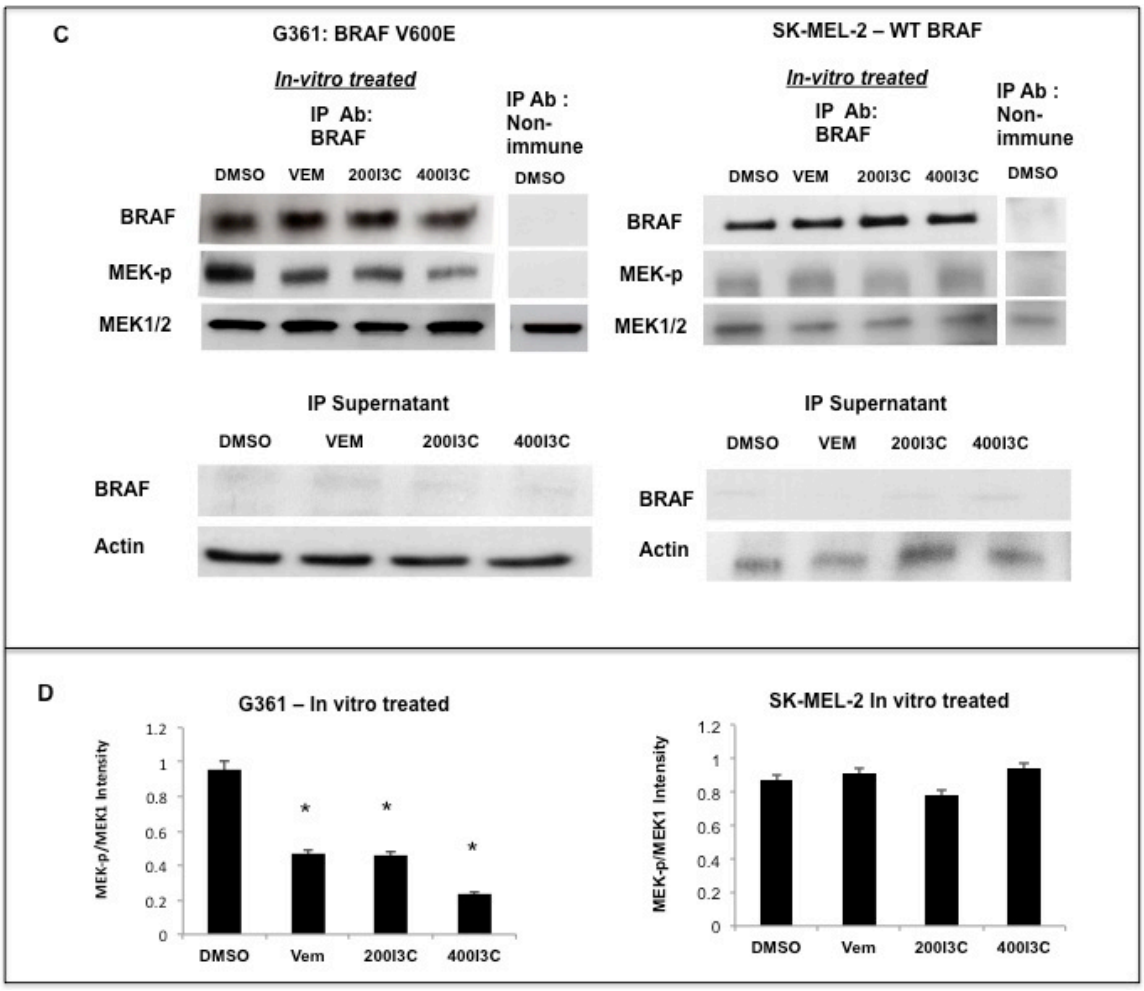
Fig 6A
I3C binding site on mutant B-Raf V600E
I3C binding site on WT B-Raf
HRD motif of catalytic loop
DFG motif
DMSO
Val 600 Glu
BRAF V600E
BRAF Wild Type
Ser674
Pro675
Leu696
Leu677
Lys697
B
G361: BRAF V600E
Cellular treated
IP Ab: BRAF
IP: Non Immune
DMSO VEM 200I3C 400I3C
BRAF
MEK-P
MEK 1/2
IP supernatant
DMSO VEM 200I3C 400I3C
BRAF
Actin
G361- Cellular treated
MEK-p/MEK1 Intensity
2.5
2
1.5
1
0.5
0
DMSO
Vem
200I3C
400I3C

Figure 6.

In silico and in vitro demonstration that I3C is an inhibitor of oncogenic BRAF- V600E enzymatic activity.

A. Predictive binding simulations of I3C interactions with mutant BRAF-V600E and wild type BRAF were analyzed using the PyMol program (left side simulation). The LigPlot program examined Van der Waals interactions (within 3.5 Å) between I3C and amino acids in either BRAF-V600E or wild type BRAF (right side simulation). *Image courtesy: Jeanne Quirit.*

B. G- 361 cells were treated with 200 μM I3C, 400 μM I3C, 10 μM Vemurafenib or with DMSO vehicle control for 48 hours. BRAF was immunoprecipitated from pretreated cell extracts, and assayed for its intrinsic enzymatic activity *in vitro* using inactive MEK as the substrate in the presence of ATP. Immunoprecipitation with non-immune IgG was used as a negative control. The level of *in vitro* generated phospho-MEK was determined by western blot analysis (left panel). The lower panels show the level of BRAF remaining in the cell extracts after immunoprecipitation by western blot analysis.


C
G361: BRAF V600E
In-vitro treated
IP Ab: BRAF
IP Ab : Non-immune
DMSO VEM 200I3C 400I3C DMSO
BRAF
MEK-p
MEK1/2
IP Supernatant
DMSO VEM 200I3C 400I3C
BRAF
Actin
SK-MEL-2 – WT BRAF
In-vitro treated
IP Ab: BRAF
IP Ab : Non-immune
DMSO VEM 200I3C 400I3C DMSO
BRAF
MEK-p
MEK1/2
IP Supernatant
DMSO VEM 200I3C 400I3C
BRAF
Actin
D
G361 – In vitro treated
MEK-p/MEK1 Intensity
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
DMSO Vem 200I3C 400I3C
SK-MEL-2 In vitro treated
MEK-p/MEK1 Intensity
1.2
1
0.8
0.6
0.4
0.2
0
DMSO Vem 200I3C 400I3C

C. BRAF-V600E was immunoprecipitated from untreated G-361 cells and incubated *in vitro* with 200 μM I3C, 400 μM I3C, 10 μM Vermurafenib or the DMSO vehicle control. BRAF-V600E enzymatic activity was assayed using the level of detected *in vitro* phosphorylation of inactive MEK as described above.

D. Wild type BRAF was immunoprecipitated from SK-MEL-2 melanoma cells and BRAF enzymatic activity post I3C treatment was analyzed as described for Fig 4C.

E. I3C regulation of BRAF enzymatic activity *in vitro* was quantified by densitometry of MEK-P and total MEK protein levels detected by western blots from three independent experiments ± SEM (*, $p < 0.01$).

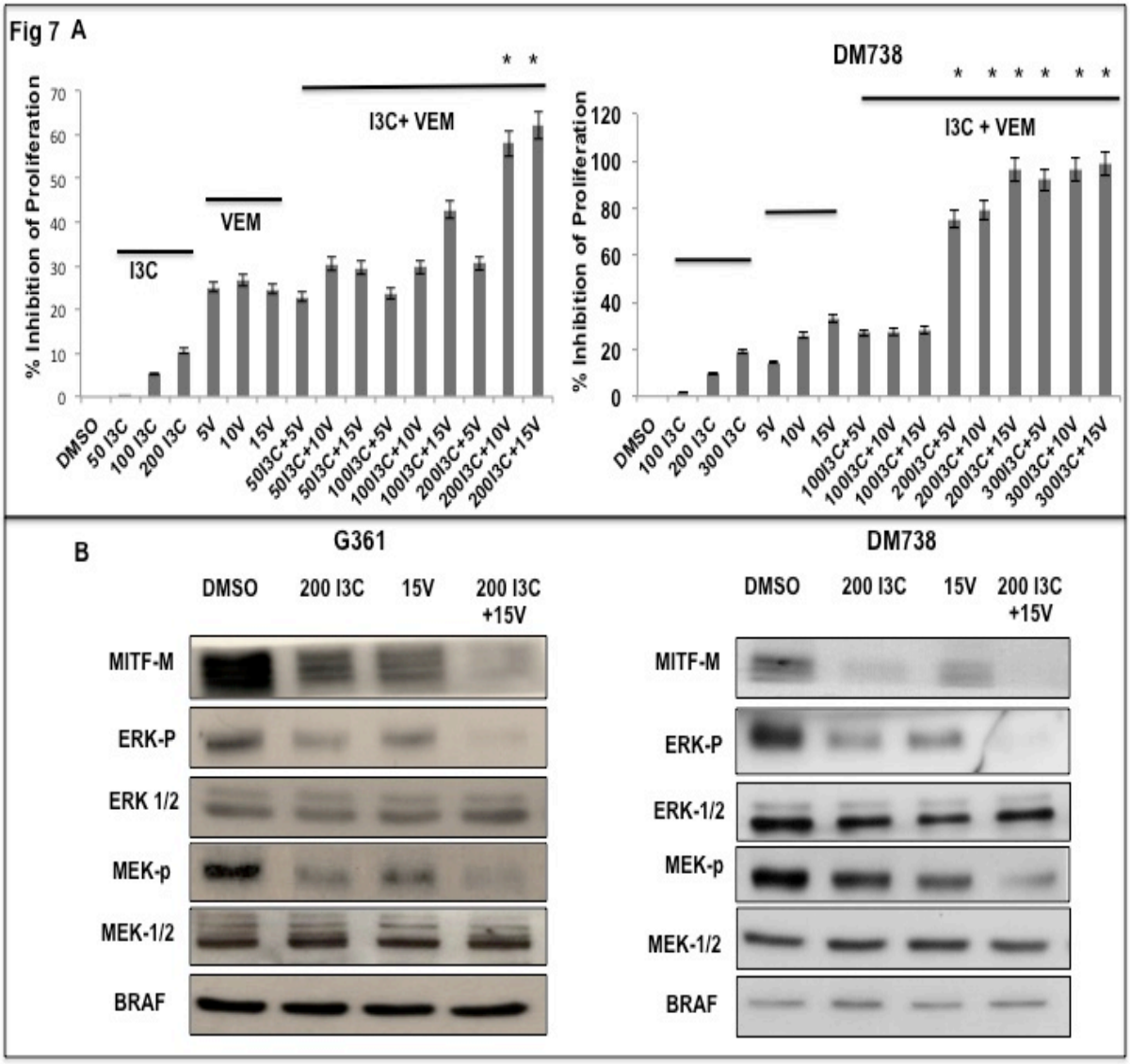


Fig 7 A
% Inhibition of Proliferation
I3C
VEM
I3C+ VEM
DMSO
50 I3C
100 I3C
200 I3C
5V
10V
15V
50I3C+5V
50I3C+10V
50I3C+15V
100I3C+5V
100I3C+10V
100I3C+15V
200I3C+5V
200I3C+10V
200I3C+15V
DM738
% Inhibition of Proliferation
I3C + VEM
DMSO
100 I3C
200 I3C
300 I3C
5V
10V
15V
100I3C+5V
100I3C+10V
100I3C+15V
200I3C+5V
200I3C+10V
200I3C+15V
300I3C+5V
300I3C+10V
300I3C+15V
B
G361
DMSO
200 I3C
15V
200 I3C +15V
MITF-M
ERK-P
ERK 1/2
MEK-p
MEK-1/2
BRAF
DM738
DMSO
200 I3C
15V
200 I3C +15V
MITF-M
ERK-P
ERK-1/2
MEK-p
MEK-1/2
BRAF

Figure 7.

Effects of combinations of I3C and Vermurafenib on melanoma cell proliferation and BRAF-V600E signaling.

A G-361 or DM-738 cells were treated with the indicated combinations of I3C and Vermurafenib for a suboptimal time of 24 hours and inhibition of proliferation was monitored using a CCK-8 assay. The results represent the average of three independent experiments with a mean ± SEM (*, $p < 0.01$) shown in the bar graphs.

B. G-361 cells and DM-738 cells were treated with combinations of 200 μM I3C and/or 15 μM Vermurafenib for 24 hours and the levels of the indicated proteins determined by western blots on total cell extracts.

RESULTS

I3C inhibits proliferation and down-regulates MITF-M levels in oncogenic BRAF-expressing human melanoma cells in culture and in vivo.

Because MITF-M plays a central role in the regulation of melanoma cell proliferation and differentiation [25], we initially examined whether I3C disrupts the *in vivo* production of this transcription factor in human melanoma cell-derived tumor xenografts. G-361 melanoma cells expressing oncogenic BRAFV600E and sensitive to the anti-proliferative effects of I3C [20,21] were subcutaneously injected into NIH III athymic mice to form tumor xenografts. The mice were injected with either I3C (200 mg/kg body weight) or DMSO vehicle control throughout a four-week course. I3C strongly inhibited tumor growth that was noticeable within the first week of injections and sustained over the entire time course (Fig 1A). Immunofluorescence analysis of tumor xenograft sections demonstrated that concomitant with the *in vivo* inhibition of tumor growth, I3C injected animals showed a strong down-regulation of MITF-M protein relative to vehicle treated control animals (Fig 1B, left panels). Similar down regulation of MITF-M protein was observed by immunofluorescence analysis of cultured G-361 melanoma cells treated for 72 hours with or without 200 μM I3C (Fig 1B, right panels), which is the optimal I3C concentration for the anti-proliferative effect in cultured human cancer cells [26]. This result indicated that the I3C down regulation of MITF-M levels observed *in vivo* is a direct effect in the melanoma cells, rather than an indirect consequence of other tissues conceivably producing a factor in response to I3C that acts on the melanoma-derived tumor xenografts.

The potential effects of I3C on melanoma cell proliferation and production of MITF-M was tested in five different human melanoma cell lines, with distinct mutagenic profiles including G-361 cells. Each of the cell lines was treated for 72 hours with or without 200 μM I3C, and cell proliferation determined using a CCK-8 proliferation assay. Most sensitive to the I3C anti-proliferative effects were G-361, DM738 and RPMI-7951 cells, which harbor the BRAF-V600E oncogenic mutation, although each cell line differs with respect to their NRAS, PTEN and P53 genotypes (Fig 1C). Western blot analysis of melanoma cells treated with varying concentrations of I3C for 48 hours showed that I3C strongly down-regulated MITF-M levels in G-361 and DM738 cells at approximately the same concentration range (Fig 1D, upper and middle set of panels). Consistent with the regulators CDK2, CDK4, cyclin D1 and p21 being transcriptional target genes of MITF-M, the I3C inhibition of MITF-M production dose-dependently and temporally correlated with the G1 cell cycle arrest and down-regulated levels of cell cycle genes in BRAF-V600E expressing melanoma cells (Fig 6 and Fig 7). In contrast, wild type BRAF expressing melanoma cell lines, SK-MEL-24 and SK-MEL-2, were significantly less sensitive to the anti-proliferative effects of I3C (Fig 1C), SK-MEL-2 expressed high levels of MITF-M protein at all concentrations of I3C (Fig 1D, lower set of panels) and did not down regulate MITF-M targets or display a G1 cell cycle arrest in the presence of I3C (Fig 6 and Fig 7).

I3C induces G1 cell cycle arrest in sensitive cells by downregulating key cell cycle regulators.

As shown in Figure 6, I3C dose dependently induced a G1 cell cycle arrest of BRAF-V600E expressing G361 and DM738 melanoma cells. In contrast, I3C had no anti-proliferative effect on wild type BRAF expressing SK-MEL-2 cells at all tested concentrations of this indolecarbinol compound.

MITF-M maintains melanoma cells in a highly proliferative state in part by promoting expression of several cell cycle target genes such as CDK2, CDK4 and Cyclin D1 and inhibiting expression of the p21 CDK inhibitor. Therefore, we tested whether I3C can induce a G1 cell cycle arrest in melanoma cells in which I3C treatment results in the loss of MITF-M protein levels. The BRAF-V600E-expressing G361 and DM738 cells and the wild type BRAF-expressing SK-MEL-2 cells were treated with or without 200 μM I3C over a 72 hour time course. At each time point, the nuclear DNA content was analyzed by flow cytometry analysis of propidium iodide stained nuclear, and the production of MITF-M and its cell cycle gene targets determined by western blot analysis. In both G361 and DM738 melanoma cells, which express BRAF-V600E, I3C rapidly down regulated the levels of MITF-M protein and reduced the levels of its positively regulated target genes CDK2, CDK4 and Cyclin D1. I3C treatment also stimulated the levels of the p21 CDK inhibitor, which is negatively regulated by MITF-M (Figure 7A, left and middle panels). In a manner that correlated with the loss of MITF-M protein, I3C significantly stimulated the number of cells with a G1 phase DNA and reduced the number of S phase cells, indicative a G1 cell cycle arrest (Figure 7B, left and middle panels). In contrast, in wild type MITF-M expressing SK-MEL-2 melanoma cells, I3C treatment triggered a modest proliferative response as shown by the loss of G1 phase cells and enhanced levels S phase cells along with the stimulated production of MITF-M and its CDK4 target gene (Figure 7A and Figure 7B, right panels). No changes in the levels of CDK2, Cyclin D1 and p21 were observed during the time course of I3C treatment of SK-MEL-2 cells.

Expression of exogenous MITF-M rescues the I3C down-regulation of the MITF-M target genes CDK2 and CDK4 and attenuates the I3C proliferative arrest of melanoma cells.

Exogenous MITF-M was expressed in G-361 melanoma cells to functionally test whether the I3C induced down regulation of MITF-M is required to mediate the I3C anti-proliferative response. As shown in Figure 2A, cells transfected with an MITF-M expression vector (pCMV-MITF) displayed high levels of MITF-M protein in the presence or absence of I3C, whereas, MITF-M protein levels were strongly down regulated by I3C in empty expression vector (pCMV) transfected cells or in untransfected cells. Western blots further revealed that the I3C down-regulation of two MITF-M target genes, CDK2 and CDK4, was rescued in cells expressing exogenous MITF-M protein (Figure 2A). Consistent with a critical role of MITF-M down-regulation for the I3C anti-proliferative effects, expression of exogenous MITF-M protein strongly attenuated this process in cells treated with I3C for 24 hours (Figure 2B). Empty vector transfected or untransfected melanoma cells showed only a 60% proliferative arrest because of the submaximal incubation time of 24 hours employed to ensure maximal expression of exogenous MITF-M.

I3C down regulates MITF-M expression by disrupting MITF-M promoter activity and Brn2 interactions with the MITF-M promoter

The effects of I3C on MITF-M transcript levels was examined in BRAF-V600E expressing G-361 and DM738 cells as well as wild type BRAF-expressing SK-MEL-2 cells treated for a 72 hour time course with or without 200 μM I3C. RT-PCR analysis of total RNA at each time point revealed that I3C strongly down regulated MITF-M transcript levels in both the BRAF-V600E expressing melanoma cell lines, which accounts for the loss of MITF-M protein levels (Fig 2C, upper and middle sets of panels). The G-361 cells displayed a greater loss of MITF-M transcripts compared to DM738 cells. In contrast, in the wild type BRAF-expressing SK-MEL-2 cells, I3C induced a modest increase in MITF-M transcript levels (Fig 2C, lower set of panels). Consistent with the loss of MITF-M transcripts, transient transfection of a -333/+120 MITF-M promoter-luciferase reporter plasmid (WT) revealed that I3C strongly down regulated MITF-M promoter activity (Fig 2D). This MITF-M promoter fragment contains a consensus site for the melanoma specific transcription regulator BRN2 (N-Oct3) at -50 to -36, which functions downstream of the oncogenic BRAF signaling pathway to maintains high levels of melanoma MITF-M [3]. Mutation of the Brn2 consensus site (BRN2 Mut) prevented the I3C down regulation of MITF-M promoter activity (Fig. 2D), which directly implicates the involvement of BRN2 in the I3C inhibition of MITF-M promoter activity.

Chromatin immunoprecipitation (ChIP) assay demonstrated that I3C treatment disrupted binding of endogenous BRN2 to the MITF-M promoter. G-361 melanoma cells were treated with or without I3C for 48 hours, and the genomic fragments cross-linked to protein were immunoprecipitated with anti-BRN2 antibodies or with an IgG control antibody. As shown in Figure 2E, PCR analysis using primers specific to the BRN2 binding site in the MITF-M promoter revealed that I3C significantly down regulated endogenous BRN-2 interactions with this promoter. One percent input was used as a loading control. Immunofluorescence of G-361 melanoma cells treated with or without 200 μM I3C for 48 hours revealed that I3C prevented the nuclear localization of Brn2 and thereby prevents accessibility of this transcription factor to the MITF-M promoter. As shown in Figure 2F (upper panel), in vehicle control DMSO treated cells, BRN2 is highly concentrated in the nucleus, whereas, treatment with I3C disrupted the nuclear localization of BRN2 with this protein remaining diffusely localized throughout the cytoplasm (Figure 2F, lower panel).

I3C disrupts the oncogenic BRAF signaling pathway

To assess the potential effects of I3C on the immediate down stream effectors of BRAF, signaling namely MEK and ERK/MAPK, melanoma cells were treated with or without I3C over a 72 hour time course and protein levels of each signal transduction component was examined by western blots. In the BRAF-V600E expressing G-361 and DM738 cells, I3C strongly down regulated the active phosphorylated forms of MEK and Erk/MAPK with no corresponding changes in the level of total MEK or Erk/MAPK protein respectively (Fig. 3A Left and Middle panels). The total levels of BRAF-V600E protein and the control protein Hsp90 remained unchanged. In contrast, I3C had no effect on the levels of phosphorylated MEK or phosphorylated Erk/MAPK in wild type BRAF expressing SK-MEL-2 cells (Fig. 3A Right

panel), which likely accounts for the inability of I3C to down-regulate MITF-M expression in this melanoma cell line (Fig 1D). The *in vivo* effect of I3C on oncogenic BRAF signaling was analyzed by immunofluorescence on sections from G-361 cell-derived tumor xenografts. Consistent with the cellular observations, I3C strongly down regulated the level of phosphorylated Erk/MAPK with no effect on the total levels of Erk/MAPK protein in the tumor xenografts (Fig 3B).

In a complementary pharmacological approach, G-361 cells were treated for 48 hours with or without either 10 μM Vemurafenib, a clinically used BRAF-V600E specific inhibitor [27], or 10 μM UO126, a selective inhibitor of MEK1/2 activity [28]. A parallel set of cells were treated with 200 μM I3C and the effects compared to each inhibitor. I3C, Vemurafenib and U0126 each down regulated the levels of phosphorylated Erk/MAPK, MITF-M and CDK4 protein (Fig 3C). The Vermurafenib effects on MITF-M protein in melanoma cells is the first such observation for either pharmacological inhibitor. Taken together, these results indicate that in melanoma cells I3C disrupts oncogenic BRAF signaling at or upstream of MEK activation.

In silico modeling predicts a stable I3C binding site specific to the oncogenic BRAF-V600E

To initially test the possibility that I3C directly interacts with BRAF, computer-aided molecular binding simulations were performed that utilize the known 3-D crystal structures of the mutant and wild type BRAF proteins and the chemical structure of I3C (Fig.4A). Using the Hex Protein Docking software to scan the BRAF protein surfaces for potential docking sites with I3C, the molecular dynamics and thermo-stability are calculated using free energy simulations. Inspection of calculated interactions of I3C with BRAF-V600E using the LigPlot program predicts that I3C makes Van der Waals interactions (within 3.5 Å) with two critical catalytic residues Arg 575 and Asp 576 that are located in the HRD motif of the catalytic loop of BRAF-V600E. I3C is also predicted to form Van der Waals interactions with Met 620, Val 624, Ser 616, Tyr 633, Leu 577, Ala 641, Tyr 619, and Lys 578 that are generally within the catalytic loop (Fig 4A). Interestingly, the predicted I3C interacting residues in BRAF-V600E are in close proximity (less than 10 Å) to the site of the oncogenic mutation at position 600, which could explain the selectivity of I3C for the mutant BRAF protein. In contrast, simulations that used the wild type BRAF protein structure predict that I3C binds at the surface of the protein by its potential interactions with amino acid residues Leu 696, Lys 697, Ser 674, Pro 675, Leu 677), which are all located away from critical domains involved in catalysis (Fig 4A).

I3C directly inhibits catalytic activity of the oncogenic BRAF-V600E but not the wild type BRAF

The predicted I3C interactions with essential catalytic residues in the mutant BRAF-V600E protein, in particular Arg 575 and Asp 576, suggest that I3C may selectively disrupt BRAF-V600E enzymatic activity. To initially test whether I3C alters BRAF-V600E activity in cells, G-361 cells were treated for 48 hours with the DMSO vehicle control, 200 μM I3C, 400 μM I3C or with 10 μM Vemurafenib, the clinically employed BRAF inhibitor. The BRAF-V600E protein was pulled down from each of the cell extracts with anti-BRAF antibodies and the kinase

activities assayed *in vitro* by adding ATP and inactive MEK protein as the BRAF substrate. After 30 min incubation, the levels of phosphorylated MEK protein in the reaction mixture was assessed using western blots to measure of BRAF-V600E activity. As shown in Figure 4B, cellular treatment with either I3C or Vemurafenib significantly inhibited BRAF-V600E enzymatic activity as shown by the loss of phosphorylated MEK, whereas, in the absence of either compound, immunoprecipitated BRAF-V600E was highly active (Fig 4B, IP:BRAF panels). The levels of BRAF-V600E, MEK and the Hsp90 gel loading control in each of the reaction mixtures remained constant.

The *in vitro* kinase assay was further used to assess the potential direct effects of I3C on BRAF enzymatic activity. BRAFV600E or wild type BRAF protein were first immunoprecipitated from untreated G-361 or SK-MEL-2 melanoma cells respectively and then pre-incubated for 2 hours with the DMSO vehicle control, 200 μM I3C, 400 μM I3C or 10 μM Vemurafenib. Subsequently inactive MEK protein and ATP were added to *in vitro* reactions and after 30 min the level of BRAF activity was assessed by western blots to determine the level of phosphorylated MEK protein. As shown in Figure 4C (left panels), I3C strongly down regulated BRAF-V600E activity at levels comparable to the well-characterized BRAF-V600E inhibitor Vemurafenib. In contrast, *in vitro* incubations containing either I3C or Vemurafenib had no effect on wild type BRAF enzymatic activity (Fig 4D, right panels). The relative BRAF activities generated under each condition were quantified by densitometry of the amount of phosphorylated MEK detected in the western blots (Fig 4E). The observed BRAF-V600E kinase activity was dependent on the use of anti-BRAF antibodies in the original immune isolations because no phosphorylated MEK was observed when non-immune antibodies were used for the immunoprecipitations (Fig 4B, Fig 4C and Fig 4D, right panels). Furthermore, the post-pull down supernatant fractions were devoid of any residual BRAF protein showing that the BRAF immunoprecipitations quantitatively brought down all of the BRAF protein in each cell extract (Fig 4B, Fig 4C and Fig 4D, lower panels). Taken together, these results demonstrate that I3C functions in human melanoma cells as a direct and selective inhibitor of BRAF-V600E enzymatic activity.

Anti-proliferative effects of combinations of I3C and Vemurafenib in oncogenic BRAF-V600E expressing melanoma cells.

Given that I3C and Vemurafenib have different binding sites on the oncogenic BRAF, we tested whether a combination of I3C and Vemurafenib could induce a stronger anti-proliferative response in sensitive melanoma cells compared to each compound alone. BRAF-V600E expressing G-361 and DM738 melanoma cells were treated with different concentration combinations of I3C and Vemurafenib for a submaximal response time of 24 hours and cell proliferation was determined by CCK-8 assay. As shown in Figures 5A and 5B, a combination of I3C and Vemurafenib more effectively inhibited the proliferation of both melanoma cell lines compared to the effects of either I3C or Vemurafenib alone. The effects of combinations of 200 μM I3C and 15 μM Vemurafenib on BRAF-V600E signaling was examined in G-361 and DM738 melanoma cells. Western blot analysis of cell extracts of both cell lines after 24 hours treatment showed that a combination of I3C and Vemurafenib down-regulated MITF-M, phosphorylated Erk/MAPK and phosphorylated MEK to a significantly greater extent compared

to the individual effects of either I3C or Vemurafenib (Fig 5C and 5D). Thus, the combinational inhibitory effects of I3C and Vemurafenib on melanoma cell proliferation mirrored the down regulation of MITF-M levels.

DISCUSSION

This study establishes that I3C directly and selectively inhibits the enzymatic activity of oncogenic BRAF, but not of the wild type BRAF, thereby identifying BRAF-V600E as a new I3C target protein. The selective targeting of oncogenic BRAF by I3C accounts for BRAF-V600E expressing melanoma cells being highly sensitive to I3C anti-proliferative effects, whereas, in contrast wild type BRAF expressing cells are relatively insensitive to I3C. Because oncogenic BRAF-V600E is a key driver mutation in human melanomas, several classes of BRAF inhibitory compounds have been developed as melanoma targeted therapies, such as Vemurafenib and Dabrafenib *to treat nonresectable or metastatic melanoma [28, 29]. However, the long-term prognosis of patients treated with oncogenic BRAF inhibitors is poor because of the development of acquired drug resistance, and an increased risk for cancerous or precancerous non-melanoma skin lesions [30, 31, 32]*. Among the reported mechanisms of acquired resistance are reactivating mutations in MEK or higher expression levels of Erk/MAPK that can overcome the inhibition of oncogenic BRAF [33]. Parallel activation of the PI3-kinase/Akt pathway and up-regulating mutations in MITF-M has also been observed in melanoma tumors resistant to oncogenic BRAF inhibitor therapy [34]. In this regard, combinations of the BRAF inhibitor Dabrafenib, the MEK inhibitor Trametinib and the Akt pathway inhibitor GSK2141795B were shown to delay the emergence of drug resistance in melanoma cells [35]. We previously demonstrated in melanoma cells that I3C interacts with NEDD4-1 ubiquitin ligase and stabilizes higher levels of the wild type PTEN tumor suppressor protein, which leads to the down-regulation of active Akt-1 [20]. Because I3C can disrupt both oncogenic BRAF and Akt-1 signaling in melanoma cells through direct target protein interactions, and also cooperates with Vemurafenib in anti-proliferative signaling, an intriguing clinical possibility is that a combination of I3C and a BRAF inhibitor will effectively disrupt melanoma proliferation as well as delay or prevent the onset of acquired drug resistance in oncogenic BRAF-expressing melanoma cells.

MITF-M plays a central role in melanoma proliferation and differentiation [36], and cellular variations in MITF-M levels and/or function correlate with the efficacy of and resistance to anti-melanoma therapeutics [37]. Several studies have shown that MITF-M expression can be regulated transcriptionally in melanocytes and/or melanomas through different transcription factor interactions with the MITF-M promoter including that of CREB, Sox10, LEF and Brn2 [38]. A key consequence of the I3C inhibition of oncogenic BRAF-V600E activity is *the loss of Erk/MAPK signaling and disruption of BRN2 nuclear localization, which in turned prevented accessibility to the MITF-M promoter. As a result, I3C down regulated MITF-M promoter activity, accounting for reduced MITF-M transcript and protein levels as well as the subsequent loss of MITF-M target gene expression such as CDK2, CDK4 and cyclin D1*. Similar to the ability of I3C to inhibit BRAF-V600E signaling, treatment of melanoma cells with Vemurafenib or with the MEK inhibitor U0126 down regulated MITF-M protein levels and of phosphorylated Erk/MAPK.

I3C represents a structurally unique small molecule inhibitor of oncogenic BRAF enzymatic activity. In the BRAF-V600E protein, the salt bridge that forms between Glu600 and Lys507 stabilizes the activation loop in the 'DFG-in' conformation, allowing this domain to form

hydrophobic interactions with the HRD motif in the catalytic loop [39], thus rendering the oncogenic mutant protein constitutively active. Upon ATP binding to BRAF, Asp 576 of the catalytic loop acts as a base catalyst to activate a substrate hydroxyl group and thus increases its nucleophilicity. *In silico* simulations predict that I3C stably interacts with Arg 575 and Asp 576, as well as with several other spatially adjacent amino acid residues, in BRAF-V600E, whereas the same simulations predict that I3C interacts with the wild type BRAF protein on a surface outside the catalytic loop. Thus, I3C's predicted association with essential catalytic amino acid residues may conceivably prevent the ability of BRAF-V600E to kinetically drive the phosphorylation reaction and ultimately displaces the HRD motif into positions that are not suited for catalysis. These *in silico* predictions are substantiated by our *in vitro* observation that I3C directly inhibits BRAF-V600E enzymatic activity without disrupting the catalytic activity of the wild type BRAF. Interestingly, the *in silico* predicted I3C site on the catalytic loop is distinct from the Vemurafenib binding site in the cleft between the N and C lobes of the kinase domain near the hinge region, which allows Vemurafenib to be anchored stably at the adenine-binding region of the ATP pocket [40]. The different binding sites within BRAF-V600E may explain the ability of combinations of I3C and Vermurafenib to cooperatively disrupt BRAF-V600E signaling leading to the loss of MITF-M expression.

Our cellular and *in vitro* observations were further substantiated *in vivo* in tumor xenografts derived from oncogenic BRAF-expressing melanoma cells in athymic mice. Injections with I3C strongly inhibited the growth rate of the tumor xenografts and down regulated levels of MITF-M protein and phosphorylated Erk/MAPK. These *in vivo* results implicate I3C and its highly potent derivatives [41] as a new class of potential therapeutic compounds for treatment of BRAF-V600E expressing melanomas. It is tempting to consider that I3C-based therapeutics could also potentially be useful to treat other types of human cancers expressing BRAF mutations, with minimal side effects because this natural indolecarbinol has been reported to be well tolerated in clinical trials of cancer patients [42]. Interestingly a wide variety of human cancers express an activating mutation in BRAF such as renal cancer [43], non-Hodgkin's lymphoma, colorectal cancer [44,45], papillary thyroid carcinoma [46,47], adenocarcinoma of the lung [48] and non-small cell lung cancer [49]. The V600E mutation is reportedly a driver mutation in 100% cases of hairy cell leukemia [50]. Given that I3C can inactivate the function of such a key oncogenic molecule its therapeutic potential in human Oncology is immense.

Additionally the current trend in the field of melanoma targeted therapy is to combine two or more anti-cancer drugs into a "cocktail" to bring about more robust anti-proliferation and sustained progression free survival. Efforts are being made to either 'hit the pathway harder' or 'hit the pathway at multiple levels' or to ' hit pathways' to counter development of drug resistance. Superior outcomes of dual BRAF and MEK inhibition in terms of lower hazard ratios, longer progression free survival, higher overall survival in patients with acquired drug resistance as well as substantially improved complete response rates even in patients without drug resistance and reduced frequency of cutaneous side effects have established combination therapy to be great new strategy to treat melanoma [51]. Prompted by positive results from Phase III clinical trials, the FDA recently approved the clinical use of a combination of Dabrafenib (BRAF Inhibitor) and Trametinib (MEK inhibitor) in patients with BRAF V600E/K mutations [52,53]. In this context the current study shows that I3C with its ability to cooperate with a clinically used competitive inhibitor of BRAF and its ability to simultaneously target multiple oncogenic

pathways (Chapter 2 – PTEN/Akt pathway inhibition) with high clinical tolerance is a promising candidate for use in drug cocktails for melanoma targeted therapy.

CHAPTER 2

Indole-3-carbinol disrupts NEDD4-1-dependent proteasomal degradation of the wild type tumor suppressor PTEN triggering an anti-proliferative response in human melanoma cells

ABSTRACT

The wild type tumor suppressor protein PTEN is absent, inactivated or expressed at low levels in 10-30% of human melanomas. Low PTEN levels show high positive correlation to melanoma genesis, progression as well as refractoriness to BRAF inhibitor therapy. I3C induced a G_1-phase cell-cycle arrest and apoptosis by stabilization of PTEN in cell lines that express the wild type protein. Melanoma cells displaying a null PTEN phenotype or expressing mutant PTEN were resistant to the anti-proliferative effects of I3C. In G-361 melanoma cells, expressing wild type PTEN, I3C disrupted the NEDD4-1 E3 ubiquitin ligase dependent ubiquitination and proteasomal without altering PTEN transcript levels. Knock down of PTEN protein levels by interfering RNA prevented the induction of apoptotic response by I3C. Furthermore, knock down of NEDD4-1 mimicked the I3C apoptotic response, stabilized PTEN protein and caused the downstream loss of phosphorylated AKT-1. Co-knockdown of PTEN and NEDD4-1 revealed that I3C- regulated apoptotic signaling through NEDD4-1 requires the presence of the wild-type PTEN protein. I3C also strongly attenuated the *in vivo* growth rate of G361 cell-derived tumor xenografts in athymic nude mice and increased PTEN protein levels in particular areas of the residual tumors. Overall, this study showed that I3C inhibits the ubiquitin ligase NEDD4-1, stabilizing the wild type PTEN tumor suppressor protein to trigger an anti-proliferative response in human melanoma cells.

INTRODUCTION

Loss of heterozygosity (LOH) at chromosome 10q coding for the PTEN gene has been reported in 30-50% melanoma cell lines and 5-20% in uncultured primary melanomas, [1] indicating the importance of its tumor suppressor function in melanoma tumorigenesis [2]. Functional studies demonstrated that ectopic expression of PTEN in PTEN-deficient melanoma cells was able to reduce tumorigenicity and metastasis confirming the involvement of PTEN in melanoma [3]. The most common melanoma associated PTEN mutations are homozygous deletions or missense, nonsense, frameshift, intronic splice mutations at position 10q 23. Mutations within PTEN tend to cluster in exon 5, which codes for the phosphatase catalytic domain. [4] Many melanomas express very low to nearly undetectable levels of the wild-type PTEN due to the loss of heterozygosity, promoter methylation, and/or alterations of protein stability [5-8]. Loss of PTEN protein expression also correlates significantly with decreased overall survival and shorter time for brain metastasis in stage III melanoma patients. [9].

While PTEN loss is mutually exclusive with the presence of an activating NRAS mutation, it is frequently detected in association with an activating BRAF mutation. A variety of genetic alterations in the tumor-suppressor PTEN (phosphatase and tensin homolog detected on chromosome 10) have been detected in human primary and metastatic melanomas including mutations or deletions [10,11]. In a Phase I clinical trial genetic abnormalities in the PTEN gene are associated with shorter progression free survival in patients treated with Dabrafenib, the BRAF inhibitor. In a Phase II trial of Vemurafenib expression of PTEN was lower in non responders compared to responders. Additionally another study reported acquisition of PTEN loss at the time of disease progression in one of 5 patients. Clinical specimens with secondary resistance to BRAF inhibitor treatment have shown overexpression and hyperactivation of receptor tyrosine kinases (RTKs) during relapse and disease progression. Emerging evidence indicates that this activation of RTKs induces a compensatory activation of the PI3K-AKT pathway.

Loss of PTEN leads to constitutive activation of the PI3K/AKT pro-proliferative pathway. PTEN normally dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3) and phosphatidylinositol 3,4- bisphosphate (PIP2) at the cell membrane [12]. PIP3 generates membrane-docking sites for both phosphotidylinositol-dependent kinase 1 (PDK1) and for the serine/ threonine protein kinase AKT-1 through their pleckstrin homology domains, where PDK1 phosphorylates and activates AKT-1.Therefore, low levels of wild-type PTEN ensures maintenance of AKT-1–mediated cell survival networks, evasion of apoptosis, and enhanced cell invasion properties of human melanoma cells [12-14].

The targeted increase in PTEN level and/or activity in melanoma cells should potentially disrupt the PDK-1– mediated activation of AKT-1 and thereby negatively regulate AKT-1 cell survival signaling [14]. Steady-state levels of PTEN protein are highly regulated by the E3 ubiquitin ligase NEDD4-1, which specifically targets PTEN for proteasomal degradation [15].

In the present study, we demonstrate that I3C selectively stabilizes PTEN protein to induce an apoptotic response in human melanoma cells that express wild- type PTEN protein. We further show that I3C disrupts the NEDD4-1–dependent ubiquitination and degradation of PTEN

protein, and directly interacts with purified NEDD4- 1 protein. Our study implicates this E3 ubiquitin ligase as a biologically significant I3C target protein in human melanoma cells and further suggests that by stabilization of PTEN protein, indolecarbinol-based compounds could potentially be utilized in new therapeutic strategies for treatment of human melanoma. The findings of this study in conjunction with the results of the Chapter 1 of this dissertation show that I3C can simultaneously target two critical oncogenic pathways involved in melanoma genesis as well as development of acquired drug resistance in melanoma.

MATERIALS AND METHODS

Melanoma cell lines G-361, SK-MEL-28, SK-MEL-30, RPMI-7951, and normal human primary epidermal mela- nocytes were all purchased from American Type Culture Collection (ATCC), and were authenticated according to the ATCC guidelines. I3C and MG-132 were purchased from Sigma-Aldrich. Two different PTEN siRNAs, two distinct NEDD4-1 siRNAs and the corresponding scram- bled siRNAs, and the HiPerFect reagents were purchased from Qiagen. Antibodies to PTEN, NEDD4-1, ubiquitin, Bcl-2, and MDM-2 were obtained from Santa Cruz Bio- technology, the antibody to Hsp90 was purchased from BD Biosciences and antibodies to PARP, cleaved PARP, cleaved caspase-3, p53, phosphor-MDM2, phospho-AKT-1, and AKT-1 were acquired from Cell Signaling Technology. The secondary anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase (HRP) were obtained from Bio- Rad Laboratories. Secondary antibodies conjugated to fluorescent probes were purchased from Molecular Probes/ Invitrogen. IDT Technologies synthesized all primers used in RT-PCR reactions.

Cell culture and indolecarbinol treatment

The G-361 melanoma cells were cultured in Modified McCoy's 5A cell media supplemented with 10% fetal bovine serum (Gemini Bio Products), 2 mmol/L L-glutamine, and 2.5 mL of 10,000 U/mL penicillin/streptomycin mixture (Gibco, Life Technologies). SK-MEL-28 and SK-MEL-30 cells were cultured in DMEM with 4.5 g/L glucose supple- mented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 2.5 mL of 10,000 U/mL penicillin/streptomycin mixture in addition to 1 of MEM nonessential amino acid (Gibco, Life Technologies). RPMI-7951 cells were cultured in DMEM containing 4.5 g/L glucose, 114 mg/L sodium pyruvate, and 2 mmol/L L-glutamine, supplemented as described above. Normal human primary epidermal melanocytes were cultured in Dermal Cell Basal Medium supplemented with the Melanocyte Growth Kit (ATCC) and 2.5 mL of 10,000 U/mL penicillin/streptomycin mixture. The cells were incubated in tissue culture dishes (Nalgene Nunc) at 37–C with controlled humidity and 5% CO_2 air content. All treatment conditions used cells at approximately 80% confluency. I3C was dissolved in 99.9% high-performance liquid chromatography (HPLC) grade DMSO (Sigma-Aldrich) and the final dilution was performed in the media aliquots used for treatment. For use of the MG-132 proteasome inhibitor, G-361 and SK-MEL- 30 melanoma cells were treated with or without 200 mmol/L I3C for 48 hours, and the cell cultures were incubated in the presence or absence of 10 mmol/L MG-132 for the last 5 hours of the treatment. The DNA content of propidium iodide–stained nuclei from 48-hour I3C-treated and untreated cells was determined by flow cytometry as previously described [16].

Western blot analysis and immunoprecipitations

Western blot analyses of samples electrophoretically fractionated on 8% to 10% acrylamide gels were carried out as previously described [15,16]. Enhanced chemiluminescence (ECL) lightening reagents were used to visualize the primary antibody–bound protein bands in nitrocellulose membranes and the results were captured on the ECL Autoradiography Film (GE Healthcare). Immunoprecipitations were carried out as previously described [17]. The cell lysates were immunoabsorbed using either mouse PTEN- specific antibodies or nonimmune antibodies bound to protein-G–coupled precleared Sepharose beads. Of note, 1% by volume of total protein extracts were separately analyzed as the input control before adding beads and antibodies. Immunoabsorbed and the input material were fractionated in a 12% polyacrylamide gel, transferred to nitrocellulose membranes, and then the samples were probed with ubiquitin- and mouse PTEN-specific antibodies. HSP90 was used as a gel-loading control for the input samples. Membranes containing the immunoprecipitated material were stripped and reprobed with a rabbit PTEN- specific antibody as a loading control for total PTEN in the immunoprecipitated samples. Negative control samples containing IgG lacked any immunoprecipitated proteins.

RT-PCR

Total RNA was extracted using the RNeasy Extraction Kit for mammalian cells (obtained from Qiagen) and spectrophotometrically quantified by absorbance at 260 nm. Reverse transcription (RT) reactions were carried out using RT-MMLV reverse transcriptase (Invitrogen) and the cDNA was used for PCR reactions using 10 pmol/ L of the following primers:

PTEN forward, CCACCAGCAGCTTCTGCC ATCTCT and reverse, CCAATT-CAGGACCCACACGACGG; GAPDH forward, TGAACGGGAAGCTCACTGG and reverse, TCCAC- CACCCTGTTGCTGTA.

PCR conditions were as follows: 30 seconds at 94–C, 30 seconds at 55–C, and 30 seconds at 72–C for 28 cycles. PCR products were electrophoretically fractionated in 1.5% agarose gels containing 0.01% Gel Red (Biotium) for DNA staining along with 1 kb plus DNA ladder and further visualized by a UV transilluminator.

siRNA transfections

Transfections with siRNA were carried out according to the Qiagen HiPerFect transfection protocol as we previously described [18,19,20]. Briefly, G-361 cells were plated at 50% confluency on 6-well plates in full cell culture media, and 150 ng of siRNA was mixed with 100 mL of media without serum or antibiotics. HiPerFect transfection reagent was then added to the siRNA suspensions. After vortexing, each mixture was incubated for 10 minutes at room temperature, and added to cells in a dropwise manner while gently agitating to ensure even distribution of the siRNA. The cells were incubated for 24 hours in full growth media, and after aspirating the media, the cells were treated in the presence or absence of 200 mmol/L I3C in full-growth media for 48 hours, with the media being replaced every 24 hours of treatment.

G-361 cell–derived tumor xenografts in athymic mice Approximately 1 million G-361 cells in a total volume of 0.1 mL of Matrigel were injected subcutaneously into each lateral flank of NIH III athymic nude mice. The resulting tumors were allowed to grow to a mean starting volume of 146 X10 mm^3. The animals were then randomized into two groups, each containing 5 mice; a vehicle control group that was treated with DMSO and an I3C-treated group that were injected daily in their scruff with 200 mg/kg body weight of I3C. The resulting tumor volumes were measured every alternate day for a period of 19 days using calipers. The tumor volumes were calculated using the standard formula: ($width^2$ length)/2, and changes in tumor volumes were calculated using the formula: 100 þ {(T_f _ T_i)/T_i 100}, where T_f is the final mean tumor volume and T_i is the initial mean tumor volume. The data represent the mean ` SEM (**, $P < 0.01$) for 5 mice per group, each with two tumors, one in each flank. At terminal sacrifice, tumor xenografts were harvested, and a portion of each tumor was fixed in 4% paraformaldehyde for 1 hour at room temperature, followed by a PBS wash and subsequently immersed in 3% sucrose overnight at 4–C. The resulting tissues were embedded in optimal cutting temperature (OCT) and 10-mm thin sections were taken for immunofluorescence studies using primary antibodies to PTEN.

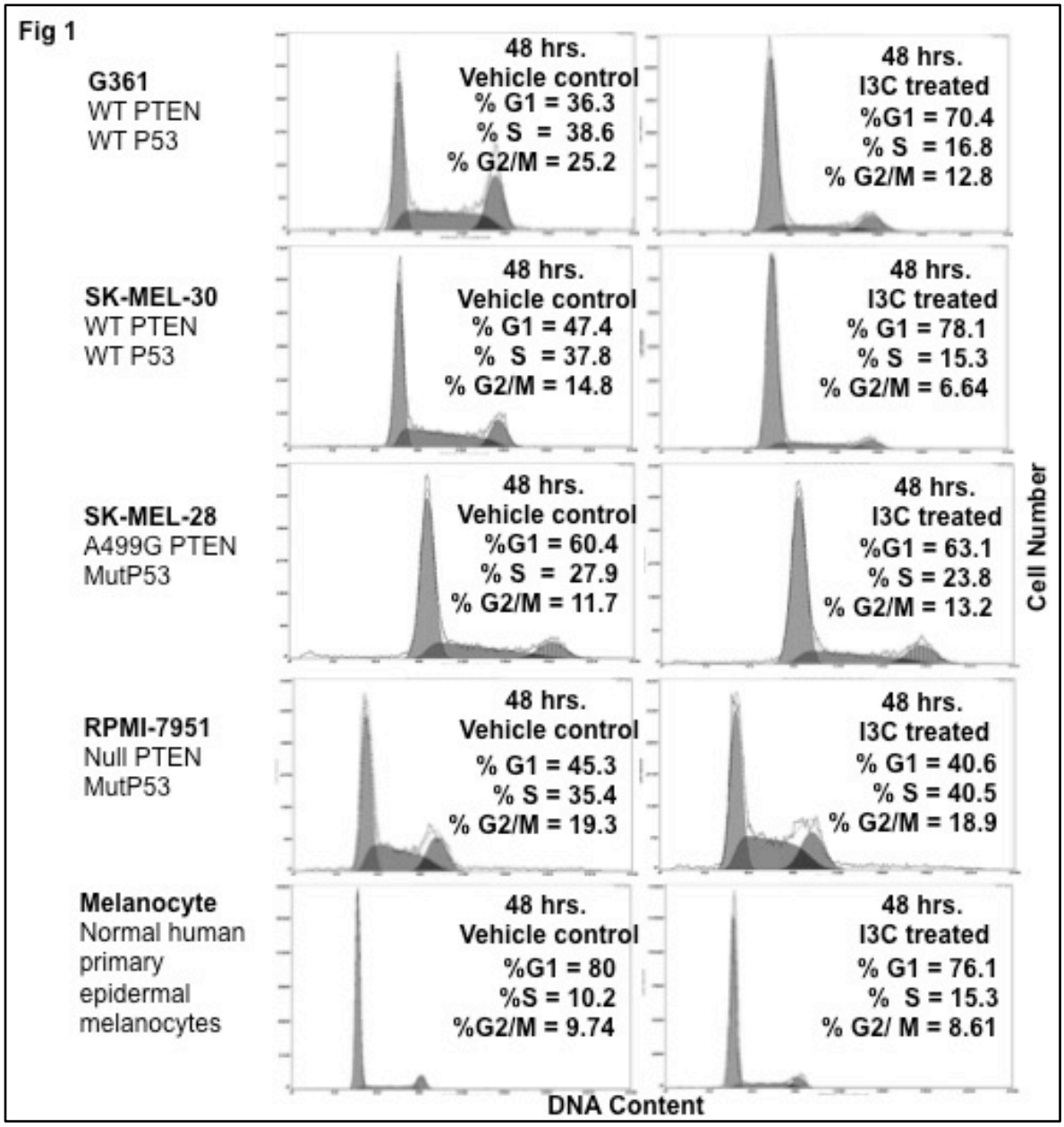


Fig 1
G361
WT PTEN
WT P53
48 hrs.
Vehicle control
% G1 = 36.3
% S = 38.6
% G2/M = 25.2
48 hrs.
I3C treated
%G1 = 70.4
% S = 16.8
% G2/M = 12.8
SK-MEL-30
WT PTEN
WT P53
48 hrs.
Vehicle control
% G1 = 47.4
% S = 37.8
% G2/M = 14.8
48 hrs.
I3C treated
% G1 = 78.1
% S = 15.3
% G2/M = 6.64
SK-MEL-28
A499G PTEN
MutP53
48 hrs.
Vehicle control
%G1 = 60.4
% S = 27.9
% G2/M = 11.7
48 hrs.
I3C treated
%G1 = 63.1
% S = 23.8
% G2/M = 13.2
RPMI-7951
Null PTEN
MutP53
48 hrs.
Vehicle control
% G1 = 45.3
% S = 35.4
% G2/M = 19.3
48 hrs.
I3C treated
% G1 = 40.6
% S = 40.5
% G2/M = 18.9
Melanocyte
Normal human
primary
epidermal
melanocytes
48 hrs.
Vehicle control
%G1 = 80
%S = 10.2
%G2/M = 9.74
48 hrs.
I3C treated
% G1 = 76.1
% S = 15.3
% G2/ M = 8.61
Cell Number
DNA Content

Figure 1.

Cell-cycle effects of I3C in human melanoma cells with different PTEN genotypes.

Cultured human melanoma cell lines (G-361 cells, SK-MEL-30 cells, SK-MEL- 28 cells, and RPMI-7951 cells) that display distinct genotypes as well as human primary epidermal melanocytes were treated with or without 200 mmol/L I3C for 48 hours. Harvested cells were stained with a hypotonic solution containing propidium iodide, and the DNA content of stained nuclei were quantified by flow cytometry analysis as described in the Materials and Methods. The histograms of representative experiments from three independent experiments are shown and the percentage of cells in the population displaying G_1, S, or G_2–M content determined for condition.

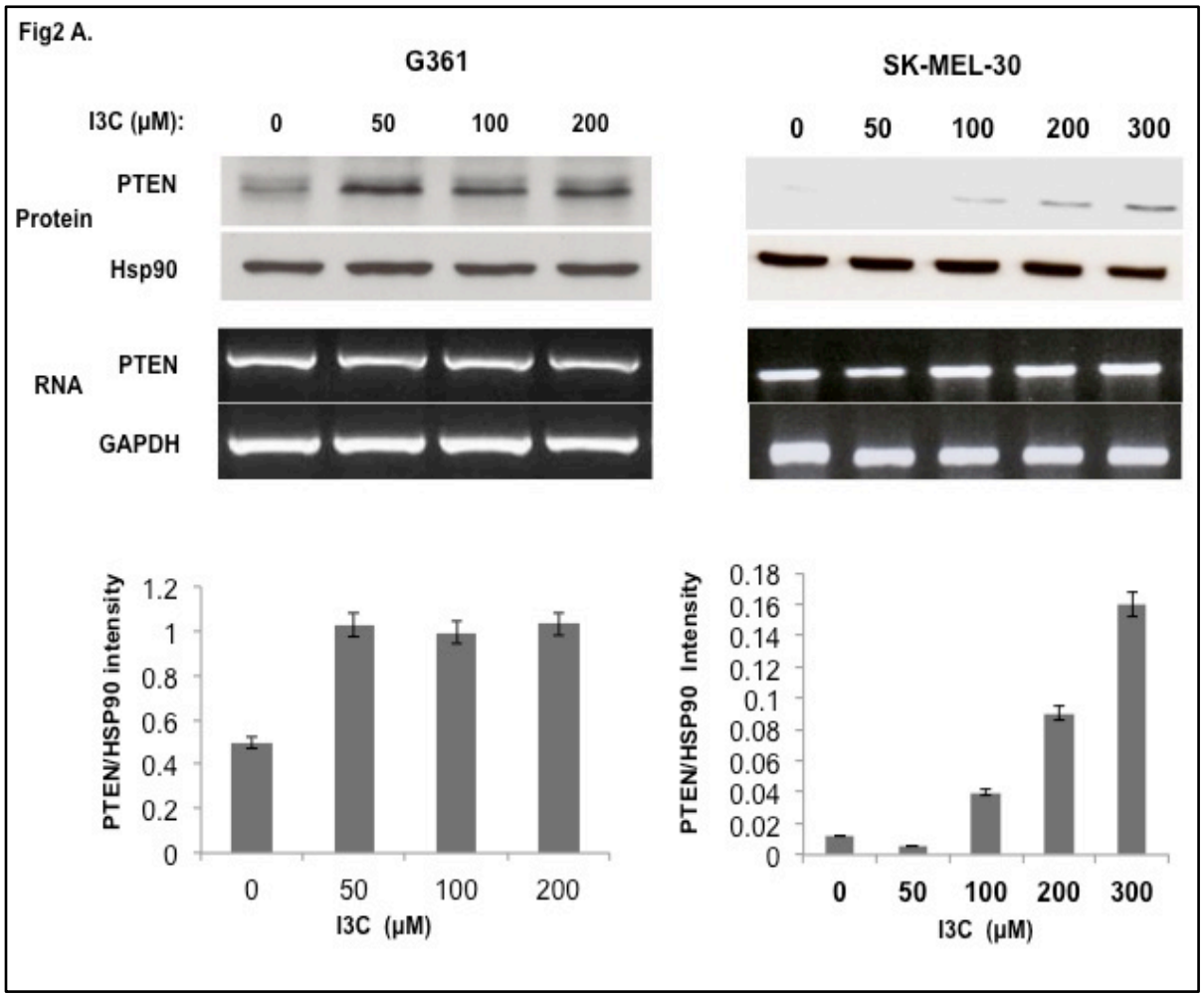


Fig2 A.
G361
SK-MEL-30
I3C (μM):
0
50
100
200
300
Protein
PTEN
Hsp90
RNA
PTEN
GAPDH
PTEN/HSP90 intensity
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
200
I3C (μM)
PTEN/HSP90 Intensity
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
50
100
200
300
I3C (μM)

Figure 2.

I3C stimulates the level of wild type PTEN protein by downregulating ubiquitination and preventing the proteasomal degradation of PTEN.

A. G-361 and SK-MEL-30 melanoma cells were treated with the indicated concentrations of I3C for 48 hours. Isolated cell lysates were fractionated by SDS–polyacrylamide gel electrophoresis and PTEN protein was monitored by Western blot analysis in comparison the HSP90 gel-loading control. Representative blots from three independent experiments are shown. Total cellular RNA was isolated, and PTEN transcript expression was determined by RT-PCR in comparison with the GAPDH constitutively expressed control transcript. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Representative gels from three independent experiments are shown. At each concentration of I3C, the relative levels of PTEN protein compared with Hsp90 were quantified by densitometry of the Western blot analyses shown in A. The average protein band intensity from three independent experiments is displayed by bar graphs.

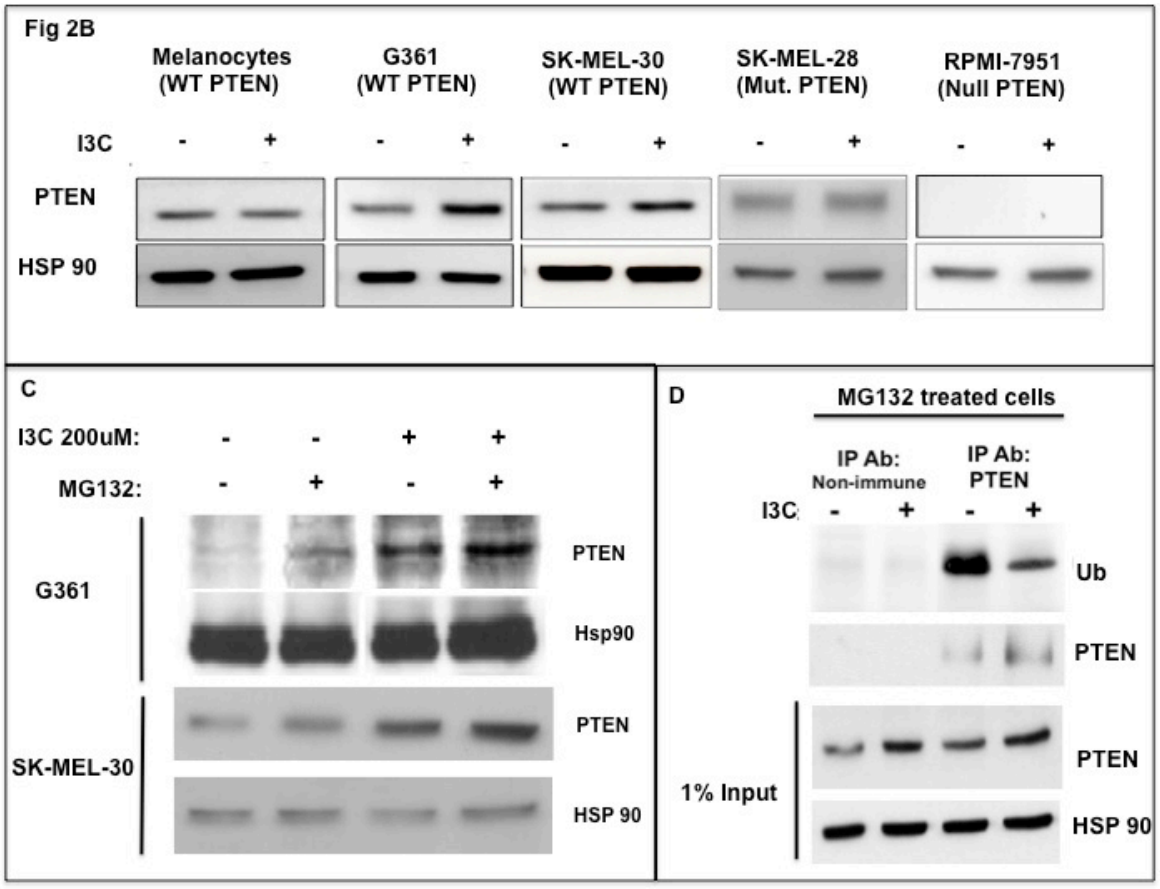


Fig 2B
Melanocytes (WT PTEN)
G361 (WT PTEN)
SK-MEL-30 (WT PTEN)
SK-MEL-28 (Mut. PTEN)
RPMI-7951 (Null PTEN)
I3C
PTEN
HSP 90
C
I3C 200uM:
MG132:
G361
SK-MEL-30
PTEN
Hsp90
HSP 90
D
MG132 treated cells
IP Ab: Non-immune
IP Ab: PTEN
I3C
Ub
1% Input

B. Human G-361 and SK-MEL-30 melanoma cells that express wild-type PTEN, human RPMI melanoma cells with a null PTEN genotype, human SK-MEL-28 melanoma cells that express an A499G mutated PTEN, and human primary epidermal melanocytes that express wild-type PTEN were treated with or without 200 mmol/L I3C for 48 hours. Total cell lysates were fractionated by SDS–polyacrylamide electrophoresis and the levels PTEN protein monitored by Western blot analysis in comparison the HSP90 gel-loading control. Representative blots from three independent experiments are shown.

C. G-361 and SK-MEL-30 melanoma cells were treated with or without I3C for 48 hours and each set of cells were incubated in the presence or absence of 10 mmol/L MG-132, a 26S-specific proteasome inhibitor, for the last 5 hours of the incubation. PTEN protein was assessed in total cell lysates by Western blot analysis in comparison with the HSP90 gel-loading control.

D. G-361 cells were treated for 48 hours in the presence or absence of I3C, and the cells were treated with 10 mmol/L MG-132 for the last 5 hours of the incubation. Cell extracts were immunoprecipitated with either PTEN-specific or nonimmune antibodies and Western blot analyses of electrophoretically fractionated samples probed with anti-ubiquitin or PTEN-specific antibodies in comparison with the HSP90 gel-loading control. The total level of PTEN protein produced in each condition is shown by the Western blot analysis of the 1% input of cell extracts. For clarity of presentation, paired gel lanes of cells not treated with the MG132 26S-proteasome inhibitor were cropped from D (top), which presents the Western blot analysis of ubiquitinated PTEN compared with the nonimmune antibody control.

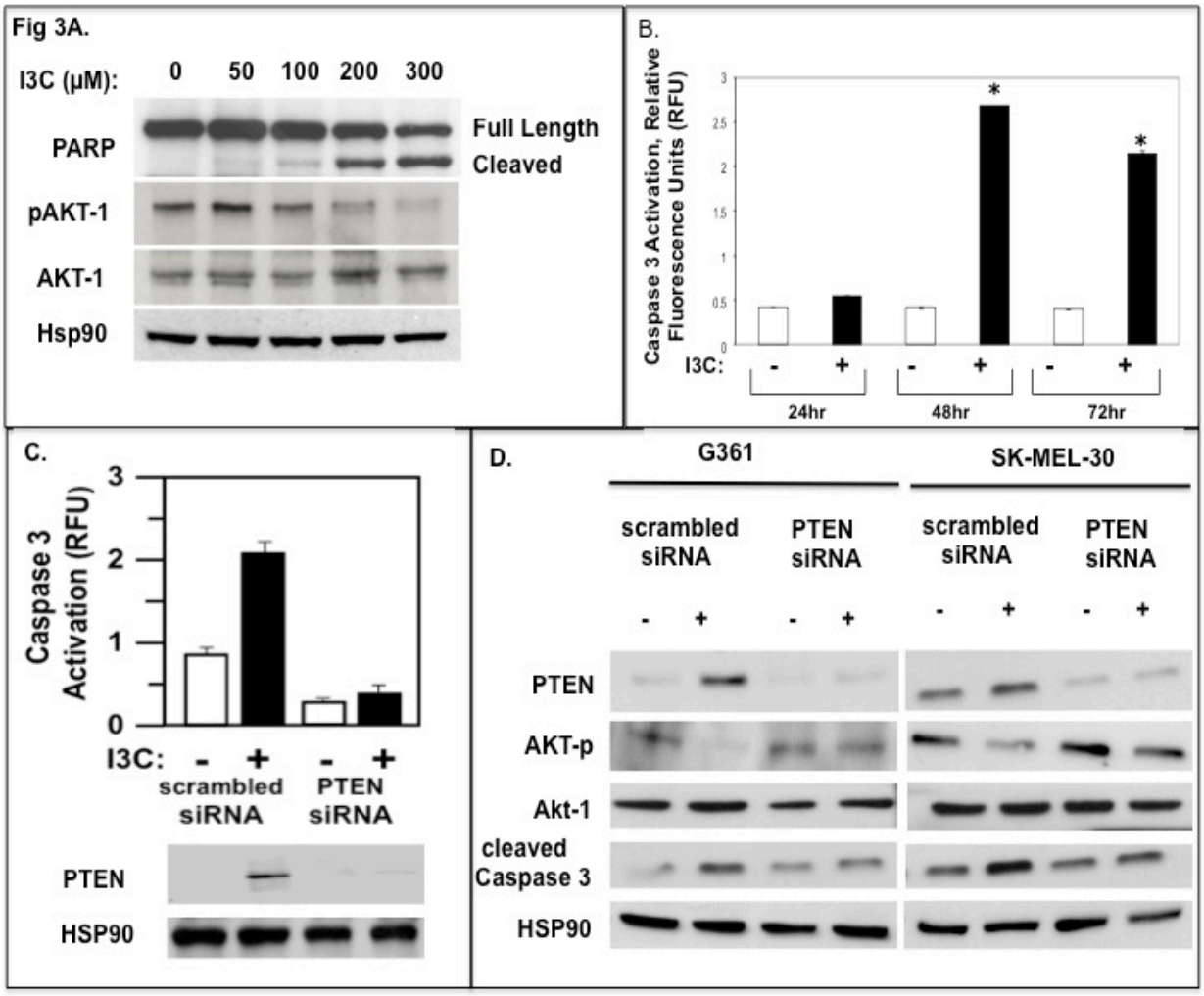


Fig 3A.
I3C (μM):
0
50
100
200
300
PARP
Full Length
Cleaved
pAKT-1
AKT-1
Hsp90
B.
Caspase 3 Activation, Relative Fluorescence Units (RFU)
I3C:
24hr
48hr
72hr
C.
Caspase 3 Activation (RFU)
I3C:
scrambled siRNA
PTEN siRNA
PTEN
HSP90
D.
G361
SK-MEL-30
scrambled siRNA
PTEN siRNA
PTEN
AKT-p
Akt-1
cleaved Caspase 3
HSP90

Figure 3.

Role of PTEN in the I3C-induced apoptosis of G361 melanoma cells.

A. G-361melanomacellsweretreatedwiththeindicatedconcentrations of I3C for 48 hours and protein levels of PARP (full-length and cleaved), phosphorylated Akt-1 (pAKT-1), total Akt-1, and the HSP90 gel-loading control were determined by Western blot analyses of electrophoretically fractionated total cell extracts.

B. G-361 melanoma cells were treated for the indicated times with or without 200 mmol/L I3C and caspase-3 quantified using the NucView488 live-cell assay. Cells treated with NucView488 probe releases a fluorescent moiety from a quenched state upon specific cleavage by active caspase-3. The results represent the mean SEM (*, $P < 0.01$) from three independent experiments.

C. G-361 cells were transfected with either scrambled siRNA or with PTEN-specific siRNA, and then treated with or without 200 mmol/L I3C for 48 hours. Caspase-3 activity was quantified using the NucView488 live-cell assay, and the results are an average of three independent experiments. Western blot analyses revealed the level of PTEN protein in cells transfected with each siRNA, and HSP90 was used as a gel-loading control.

D. G361 and SK-MEL-30 melanoma cells were transfected with a different set of PTEN and scrambled siRNA and Western blot analyses evaluated the levels of PTEN protein, phosphorylated AKT (AKT-p), total Akt-1, and cleaved caspase-3 proteins in comparison with the HSP90 gel loading control.

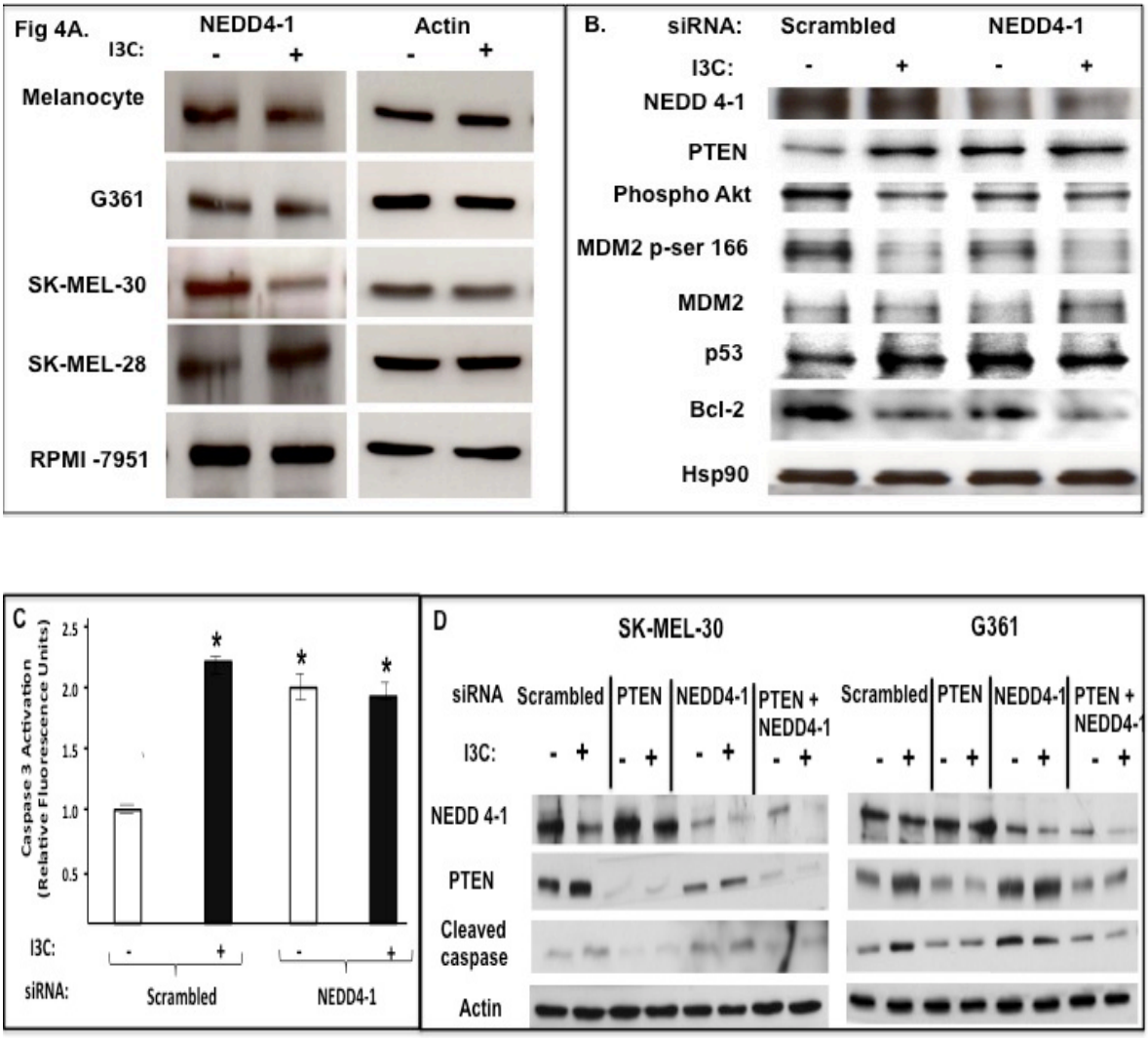


Fig 4A.
NEDD4-1
Actin
I3C:
- + - +
Melanocyte
G361
SK-MEL-30
SK-MEL-28
RPMI -7951
B.
siRNA:
Scrambled
NEDD4-1
I3C:
- + - +
NEDD 4-1
PTEN
Phospho Akt
MDM2 p-ser 166
MDM2
p53
Bcl-2
Hsp90
C
Caspase 3 Activation (Relative Fluorescence Units)
2.5
2.0
1.5
1.0
0.5
*
*
*
I3C:
- + - +
siRNA:
Scrambled
NEDD4-1
D
SK-MEL-30
G361
siRNA
Scrambled
PTEN
NEDD4-1
PTEN + NEDD4-1
I3C:
NEDD 4-1
PTEN
Cleaved caspase
Actin

Figure 4.

Role of NEDD4-1 in the I3C-induced accumulation of PTEN protein and apoptotic response.

A. Melanoma cells (G361, SK-MEL-30, SK-MEL- 28, and RPMI-7951) and melanocytes were treated with or without 200 mmol/L I3C for 48 hours and electrophoretically fractionated cell extracts analyzed by Western blot analyses probed for NEDD4-1 protein or actin.

B. G-361 melanoma cells were transfected with either scramble siRNA or with NEDD4-1–specific siRNA and then treated in the presence or absence of 200 mmol/L I3C for 48 hours. Cells were electrophoretically fractionated and Western blot analyses probed for production of PTEN, NEDD4-1, phosphorylated Akt-1, Ser-166 phosphorylated MDM2, total MDM2, p53, Bcl-2, and the HSP90 gel-loading control.

C. G361 cells were transfected with either NEDD4-1 siRNA or scrambled siRNA, and caspase-3 activity quantified in 48-hour I3C-treated and untreated cells using the NucView488 live-cell assay. The results represent the mean `SEM (*, $P < 0.01$) from three independent experiments.

D. G361 cells and SK-MEL-30 melanoma cells were transfected with the indicated combinations of NEDD4-1 siRNA and/or PTEN siRNA (different sets of siRNAs from that used in B and C) as well as with scrambled siRNA and the cells treated with or without 200 mmol/L I3C for 48 hours. Cell extracts were electrophoretically fractionated and Western blot analyses probed for NEDD4-1, PTEN, cleaved PARP, and actin. RFU (relative fluorescence units).

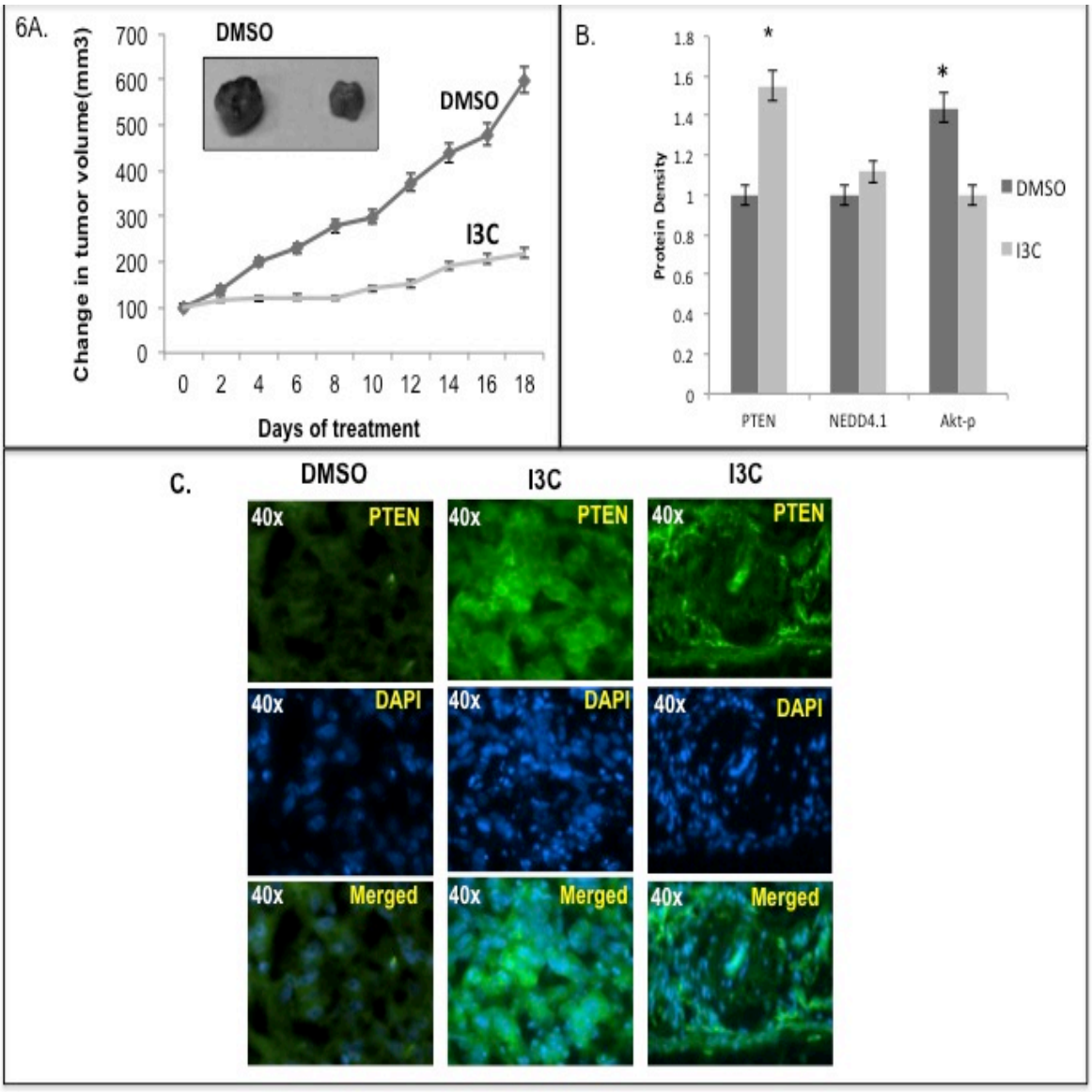

6A.
DMSO
DMSO
I3C
Change in tumor volume(mm3)
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18
Days of treatment
B.
*
*
Protein Density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PTEN
NEDD4.1
Akt-p
DMSO
I3C
C.
DMSO
I3C
I3C
40x PTEN
40x DAPI
40x Merged

Figure 5.

I3C effects on the growth as well as PTEN, NEDD 4-1, and phosphorylated Akt levels in melanoma cell derived tumor xenografts in athymic nude mice.

A. 1 million G-361 melanoma cells were implanted in each lateral flank of NIH III athymic mice, and after palpable tumors were detected (mean starting volume of 146 X10 mm^3), the mice were injected subcutaneously with either I3C (200 mg/kg body weight) or with the DMSO vehicle control over an 18-day time course. The resulting tumor volumes were measured and calculated as described in the Materials and Methods. The data represent the mean ` SEM ($P < 0.01$) for 5 mice per group, each with two tumors, one in each flank. The micrograph inset shows tumors harvested at day 18 from I3C-treated or vehicle control-treated animals.

B. at terminal sacrifice, the G-361 cell–derived tumor xenografts were harvested, and a portion of each tumor was fixed in 4% paraformaldehyde as described in the Materials and Methods. Of note, 10-mm cryostat sections from tumors excised from I3C-treated and untreated animals were analyzed for PTEN expression by immunofluorescence studies using primary antibodies to PTEN. The staining patterns were compared with DAPI staining of the cell nuclei and merged images are shown in the bottom. C, the protein densities of PTEN, NEDD4-1, and phosphorylated Akt protein in tumor xenografts from I3C-treated and untreated animals were determined by immunofluorescence staining of tumor sections. The results represent the mean `SEM (*, $P < 0.01$) of triplicate samples from three independent experiments were quantified.

RESULTS

Human melanoma cells expressing wild-type PTEN are sensitive to the antiproliferative effects of I3C

To initially determine whether human melanoma cells are sensitive to the antiproliferative effects of I3C, four human melanoma cell lines, G-361, SK-MEL-30, SK-MEL-28, and RPMI-7951, with distinct mutational profiles (described in Fig. 1), as well as human primary epidermal melanocytes, were treated with or without 200 mmol/L I3C for 48 hours, and potential cell-cycle effects were analyzed by flow cytometry of propidium iodide–stained nuclear DNA. This concentration of I3C is optimally effective in other human cancer cell lines [21,23] as the functional intracellular concentration of this indolecarbinol entering cells is less than 0.3% of the final concentration in the cell culture media [24]. As shown in Fig. 1, I3C induced a G_1 cell-cycle arrest of G-361 and SK-MEL-30 melanoma cells, both of which express wild-type PTEN and wild-type p53. In contrast, SK- MEL-28 and RPMI-7951 melanoma cell lines, which display A499G-mutant PTEN or null PTEN genotypes, respectively, were relatively unaffected by 48-hour I3C treatment (Fig. 1). SK-MEL-28 melanoma cells did show some sensitivity to I3C at time points beyond 48 hours (data not shown). Importantly, normal melanocytes were resistant to the I3C (Fig. 1), suggesting that this indolecarbinol compound triggers a melanoma-selective response.

I3C downregulates the ubiquitination and stabilizes wild-type PTEN protein levels by preventing its proteasomal degradation.

Because the strong I3C antiproliferative response correlated with the wild-type PTEN status of the melanoma cells lines, the potential effects on PTEN expression was assessed in G-361 and SK-MEL-30 cells treated over a concentration range of I3C for 48 hours. Western blot and RT-PCR analysis of PTEN protein and mRNA levels revealed that I3C strongly upregulated PTEN protein levels without altering the level of PTEN transcripts in both wild-type PTEN-expressing cell lines (Fig. 2A). The HSP90 protein and GAPDH transcript gel-loading controls remained unchanged throughout the dose response. The maximal I3C response in the G361 cells occurred at somewhat lower concentrations compared with the SK-MEL-30 melanoma cells. Densitometry of the Western blot analyses revealed that in G361 cells, treatment with 200 mmol/L I3C stimulated over a 2-fold increase in PTEN protein levels com- pared with the untreated cells with no increase in PTEN transcript levels (Fig. 2B). In SK-MEL-30 cells, the fold stimulation in PTEN protein was much higher, with 200 mmol/L I3C inducing an almost 10-fold increase in PTEN protein levels with no effect on PTEN transcript levels (Fig. 2B). In comparison with G361 and SK-MEL-30 cells, I3C had no effect on the levels of the mutant A499G-PTEN expressed in SK-MEL-28 melanoma cells, whereas PTEN expression remained ablated in RPMI-7951 cells, which display a null PTEN genotype (Fig. 2C). Normal primary melanocytes express wild-type PTEN, however, I3C had no effect on PTEN protein levels (Fig. 2C), suggesting a selective effect of I3C in melanoma cells compared with their normal cell counterpart.

To initially determine whether I3C upregulated PTEN protein levels by preventing its ubiquitin-mediated proteasomal degradation, 48-hour I3C-treated and untreated G-361 and SK-MEL-30 melanoma cells were incubated in the presence or absence of 10 mmol/L MG-132, a 26S-specific proteasome inhibitor, during the last 5 hours of the incubation. PTEN protein was assessed in total cell lysates by Western blot analysis. As shown in Fig. 2D, in the presence of MG132, PTEN protein levels were observed to increase in cells treated with or without I3C, and the combination of MG-132 and I3C resulted in an enhanced accumulation of PTEN protein. To further determine whether I3C stabilizes PTEN protein through downregulation of its ubiquitination, cells extracts from I3C-treated and untreated cells exposed to MG-132 during the last 5 hours of the incubation were immunoprecipitated with either PTEN-specific or nonimmune antibodies and Western blot analyses of electrophoretically fractionated samples probed with anti- ubiquitin antibodies. As shown in Fig. 2E, I3C strongly downregulated the level of ubiquitinated PTEN under conditions in which the level of immunoprecipitated PTEN protein increased compared with cells not treated with I3C. PTEN-associated ubiquitin was not detected in immunoprecipitations carried out with nonimmune antibodies, demonstrating the fidelity of the assay.

I3C-induced apoptosis of G-361 melanoma cells requires PTEN

The I3C-mediated stabilization of PTEN protein predicts that I3C should disrupt cell survival pathways and potentially induce apoptosis of melanoma cells in a PTEN- dependent manner. One of the key downstream effectors of PTEN is AKT-1, as PTEN dephosphorylates PIP3, and thereby prevents the PDK-1 phosphorylation and activation of AKT-1 at the plasma membrane [12-14]. G-361 melanoma cells were treated with a range of I3C concentrations for 48 hours, and the levels of cleaved poly (ADP-ribose) polymerase (PARP) molecule, which corresponds to apoptosis-related DNA repair activity, were compared with the levels of phosphorylated and total AKT-1 protein. As shown in Fig. 3A, Western blot analysis of total cell extracts using primary antibodies that detect both full-length and cleaved PARP showed that PARP cleavage was maximally induced by treatment with concentrations of I3C that stabilize PTEN and downregulate phosphorylated AKT-1. The level of total AKT-1 protein and the HSP90 gel-loading control remained relatively unchanged under these conditions.

The activity of caspase-3, an upstream regulator of PARP processing, was examined using the highly sensitive Nuc- View488 live-cell assay in which the NucView488 probe releases its fluorescent moiety from a quenched state upon specific cleavage by active caspase-3 [25]. Treatment of G-361 cells with 200 mmol/L I3C for 48 hours produced a 5- fold induction of caspase-3 activity compared with vehicle control DMSO-treated cells (Fig. 3B). The effect was slightly attenuated by 72 hours, a time point when more cells displayed cytotoxic effects (blebbing, rapid detachment from the plate surface), associated with later stages of apoptosis. To determine the role of I3C-induced stabilization of PTEN protein in melanoma cell apoptosis, initially G-361 cells were transfected with either PTEN siRNA or with scrambled siRNA, and caspase-3 activity was assessed in cells treated with or without I3C for 48 hours. As shown in Fig. 3C, siRNA knockdown of PTEN prevented the I3C- induced apoptotic response, whereas transfected scrambled siRNA had no effect on the indolecarbinol-induced apoptosis. Western blot analyses demonstrated the efficiency of the PTEN siRNA knockdown in I3C-treated cells

compared with the scrambled siRNA controls.

Because of the possibility of off-target effects of the PTEN siRNA, siRNA transfections in both G361 and SK-MEL-30 cells were carried out with a different set of PTEN-specific and scrambled siRNAs. As shown in Fig. 3D, I3C stimulated an increase in PTEN protein in cells transfected with the scrambled siRNA, whereas transfection with PTEN siRNA efficiently knocked down PTEN protein levels. In both melanoma cell lines, knockdown of PTEN disrupted the I3C regulation of phosphorylated Akt and prevented the increase in the apoptotic response based on production of the cleaved caspase-3 (Fig. 3D). The production of total Akt-1 was unaffected by either I3C treatment or the PTEN siRNA. Thus, in human melanoma cells expressing wild-type PTEN, production of PTEN is required for the I3C apoptotic response.

Direct role of NEDD4-1 in the I3C-stimulated accumulation of the PTEN protein and apoptotic response

PTEN protein stability can be regulated by the E3 ubiquitin ligase NEDD4-1, a member of neural precursor cell expressed, developmentally downregulated 4 E3 molecules, which attaches ubiquitin moieties to the PTEN protein on residues Lys 289 and Lys 48, effectively targeting it for proteasomal degradation [16]. Regardless of the PTEN status, human melanoma cells as well as normal melanocytes express generally similar levels of NEDD4-1 protein in the presence or absence I3C, although the SK-MEL-30 cells did display a reduced level of NEDD4-1 in I3C-treated cells (Fig. 4A). Because I3C downregulated PTEN ubiquitination in wild-type PTEN-expressing melanoma cells, we explored potential connections between I3C and NEDD4-1 that could regulate PTEN protein stability. To initially test the role of NEDD4-1 in the I3C-induced stabilization of PTEN protein, NEDD4-1 expression in G-361 cells was knocked down by transfection of NEDD4-1–specific siRNA. A control set of transfections was carried out with scrambled siRNA. Western blot analysis of electrophoretically fractionated cell extracts revealed that siRNA knockdown of NEDD4-1 triggered an accumulation of PTEN protein in I3C-treated and untreated cells similar to that observed after I3C treatment of cells transfected with scrambled siRNA (Fig. 4B). Thus, knockdown of the NEDD4-1 E3 ubiquitin ligase mimics the effects of I3C on PTEN protein stabilization.

Several downstream effectors of PTEN signaling that are involved in cell survival and/or apoptotic pathways were examined in melanoma cells transfected with either NEDD4-1 siRNA or scrambled siRNA. An increase in PTEN protein would be predicted to reduce the cellular level of phosphorylated AKT-1 [12-14]. As also shown in Fig. 4B, siRNA knockdown of NEDD4-1 downregulated the level of phosphorylated AKT-1 in the presence or absence of I3C to a level that closely approximates that observed in I3C-treated control cells. Activation of AKT-1 and its other isoforms has been implicated in cell survival pathways in human cancer cells through its phosphorylation of the ubiquitin ligase MDM2 [24], which in turn promotes the MDM2-mediated ubiquitination and degradation of p53, which disrupts the p53 intrinsic apoptotic program [25]. Western blot analysis further revealed that in G-361 cells transfected with scrambled siRNA, I3C induced the downregulation of Ser 166 phosphorylated MDM2 with a corresponding moderate accumulation of p53 protein. Both effects were mimicked in either I3C-treated or untreated cells transfected with NEDD4-1 siRNA (Fig. 4B). Consistent with these

effects on Ser 166 phosphorylated MDM2, I3C downregulated the level of the Bcl-2 antiapoptotic proteins (Fig. 4B), which is a downstream target of p53 [26] that has been connected to melanoma pathogenesis. Knockdown of NEDD4-1 attenuated Bcl-2 protein levels that was further reduced after I3C treatment (Fig. 4B).

To assess the potential effects of NEDD4-1 knockdown on melanoma cell apoptosis, G-361 cells were transfected with either scrambled siRNA or NEDD4-1 siRNA, and then incubated with the NucView488 fluorescent probe to assay caspase-3 activity in live cells. Knockdown of NEDD4-1 induced a strong apoptotic response in the absence or presence of I3C (Fig. 4C, NEDD4-1 siRNA) that was approximately equivalent to the I3C-nduced caspase-3 activity in control cells transfected with scram- bled siRNA (Fig. 4C).

As a complementary functional approach, NEDD4-1 production was knocked down in the absence or presence of PTEN knockdown by transfection of the corresponding siRNA in both G361 and SK-MEL-30 melanoma cells, and apoptotic signaling in cells examined in comparison those transfected with scrambled siRNA. As shown in Fig. 4D, transfection of the PTEN- and NEDD4-1– specific siRNA was highly efficient in knocking down production of the corresponding proteins. In both melanoma cell lines, knockdown of NEDD4-1 stabilized PTEN levels in the presence or absence of I3C, although compared with cells receiving scrambled siRNA, SK- MEL-30 cells transfected with NEDD4-1 siRNA expressed a reduced level of PTEN protein. Also, similar to untransfected SK-MEL-30 cells (Fig. 4A), I3C reduced the level of total NEDD4-1 protein in SK-MEL-30 cells transfected with scrambled siRNA (Fig. 4D). The I3C- stimulated production of cleaved caspase-3 in both melanoma cell lines was completely blocked in PTEN knockdown cells, whereas in NEDD4-1 knockdown cells cleaved caspase-3 was produced in I3C-treated or untreated cells (Fig. 4D). Importantly, cotransfection of siRNA for PTEN along with siRNA for NEDD4-1 in both cell lines resulted in low levels of cleaved caspase-3 in cells treated with our without I3C (Fig. 4D). Thus, I3C- regulated apoptotic signaling through NEDD4-1 requires the presence of the wild-type PTEN protein

DISCUSSION

The therapeutic enhancement of cellular PTEN levels or lipid phosphatase activity represents a potential strategy to effectively target melanoma cells that express the wild form of this tumor-suppressor protein [27, 28,12, 29]. Relatively little was known about the mechanism by which indolecarbinol compounds mediate their antiproliferative effects in human melanoma cells. We functionally established that I3C triggers an apoptotic response in human melanoma cells by increasing the level of wild-type PTEN protein resulting from its stabilization due to the disruption of the NEDD4- 1–dependent ubiquitination and 26S proteasomal degradation of PTEN protein. For example, siRNA knockdown of PTEN prevented and knockdown of NEDD4-1 mimicked the I3C stimulation of caspase-3 activity and apoptotic response, and this NEDD4-1 apoptotic response required the presence of wild-type PTEN. Furthermore, we observed that I3C directly interacts with purified NEDD4-1 protein, which identifies this E3 ubiquitin ligase as a new biologically significant I3C target protein that is critical for the control of AKT-1–mediated cell survival cascades in human melanoma cells.

We propose a direct functional connection between I3C apoptotic signaling and the disruption of NEDD4-1 E3 ubiquitin ligase targeting of PTEN protein due to the loss of NEDD4-1 activity and/or protein–protein interactions. In silico molecular modeling revealed a potential I3C binding site in the catalytic HECT domain of NEDD4-1, which is responsible for the ubiquitin ligase activity of the molecule. Consistent with the computer simulations, isothermal titration calorimetry directly demonstrated that I3C binds to purified NEDD4-1 protein with a dissociation constant of approximately 88 mmol/L. The C2 and HECT domains of NEDD4-1, which mediate the membrane localization and ubiquitin ligase activity, respectively, bind to each other, resulting in autoinhibition of NEDD4-1 activity [30]. Conceivably, I3C binding to the HECT domain could potentially inhibit NEDD4-1 activity by stabilizing the C2-HECT autoinhibitory intramolecular interactions. Furthermore, NEDD4-1 activity can be stimulated by specific activators such as the NEDD-family interacting proteins, NDFIP1 and NDFIP2, which function by binding to the WW domains of NEDD4-1 via their PY motifs, and thereby abolish the autoinhibitory interactions between the C2 and HECT domains of NEDD4-1 [30, 31]. A recent study showed that a p34 protein interacts with the WW1 domain NEDD4-1 to regulate NEDD4-1 stability and thereby PTEN ubiquitination [32]. Consistent with study, in SK- MEL-30 cells, but not the G361 cells, we observed that I3C treatment causes a moderate downregulation of NEDD4-1 protein. Hence, it is conceivable that I3C interactions with NEDD4-1 may potentially alter or disrupt protein binding to NEDD4-1 in a way that downregulates NEDD4-1 activity and/or protein levels and thereby stabilizes PTEN protein. In contrast, one study suggests that NEDD4-1 activity is dispensable for the regulation of PTEN stability and localization in mouse cells and tissue [33], although melanoma or other skin-derived transformed cells were not tested. Conceivably, different regulatory pathways could be acting on PTEN depending on the transformation state or tissue origin of the cells. Studies are currently under way to determine the precise I3C interaction site in NEDD4-1 and to access the functional consequences of the interaction.

The in silico–predicted I3C interaction site on NEDD4-1 possesses striking structural and chemical similarities with the I3C binding pocket that we characterized in neutrophil elastase, the

first identified indolecarbinol target protein [16]. Antiproliferative responsiveness of I3C in human breast cancer cells is mediated by the ability of I3C to act as a noncompetitive inhibitor of elastase enzymatic activity [16,17]. In contrast to breast cancer cells, antiproliferative signaling by I3C in melanoma cells is not mediated by the disruption of elastase cleavage of CD40 (data not shown), which implicates NEDD4-1 as a critical I3C target protein in melanoma cells expression of wild-type PTEN. Thus, an emerging concept from our previous and current studies is that I3C antiproliferative responsiveness can be triggered in different cancer cell types by the differential expression of distinct I3C target proteins.

Disruption of PTEN causing PI3Kinase/AKT activation and NRAS/BRAF mutations leading to constitutive activation of signaling via the MAPK pathway are considered two of the key drivers of melanomagenesis [9, 34, 35]. Therefore, targeting components of these pathways with either single agents or combinations have been a developing therapeutic strategy in recent years. The BRAF-mutant melanomas showed high dependency on B-RAF kinase activity and so inhibition of the oncogenic B-RAF resulted in striking reduction in tumor volume in preclinical and clinical trials [36]. On the basis of these observations, the FDA approved PLX4032/vemurafenib (Plexxicon) and dabrafenib (GlaxoSmithKline) to treat metastatic melanomas, which are two selective and potent inhibitors of the oncogenic BRAF (V600E). However, this treatment has been associated with inevitable development of rapid or acquired tumor resistance within months of the initial treatment [37]. The therapeutic potential of selective inhibitors of BRAF V600E has also been restricted by their ability to paradoxically stimulate growth in tumors harboring a wild-type BRAF [38]. Also, it has been recently reported that loss of PTEN could confer intrinsic resistance to BRAF inhibitors in melanoma cells [39], suggesting the importance of taking into account the activities of different cell signaling cascades in the treatment of human melanomas.

Our study suggests that I3C-based compounds have the potential to be developed in new therapeutic strategies for treatment of human melanoma either alone or in combination with other therapeutic compounds to target different aberrant signaling pathways. Furthermore, given the significant degree of intratumoral heterogeneity in melanoma [40] combinational therapies could conceivably be used to simultaneously target multiple cell subpopulations within the tumor. In this regard, a combination of BRAF and MEK inhibitors (dabrafenib/trametinib) is now FDA approved for patients with melanoma. Human primary epidermal melanocytes that express wild-type PTEN were not responsive to I3C, which indicates that I3C should not adversely affect normal skin cell proliferation. The precise mechanism by which normal melanocytes remain resistant to I3C are not known, and we have observed that in athymic mice, injections of I3C over 3 weeks have no apparent effects on animal weight or behavior. Clinical trials for I3C in women at risk for breast cancer have provided a valuable assessment of clinical safety and tolerated doses of I3C [41, 42]. Cytotoxicity results from this clinical trial have shown that patients are capable of receiving as high as 800 mg/kg/d I3C without any adverse side effects, which is a concentration that approximates both the 200 mmol/L I3C used in the cultured melanoma cells and the level of I3C used in the in vivo tumor xenograft experiments. Our findings implicate I3C as a potential therapeutic for melanomas that express low levels of wild-type PTEN protein in a background of BRAF V600E by disrupting the NEDD4-1–mediated ubiquitination of PTEN and enhancing the total levels of this tumor-suppressor protein. The development of structural studies on I3C interactions with its potential molecular target NEDD4-1 may provide valuable insight for the design of highly customized I3C derivatives that would more efficiently stabilize PTEN at lower doses and maximize the potential therapeutic effe

CHAPTER 3

1-benzyl Indole-3-carbinol, a potent Indolecarbinol analog inhibits Wnt signaling to downregulate MITF-M expression and cause antiproliferation in human melanoma

ABSTRACT

1-benzyl Indole-3-carbinol (I3C), a synthetic Indolecarbinol analog shows anti-proliferative effect in melanoma cells comparable to the clinically used drug Vemurafenib, but at >10-fold lower concentration than the parent compound I3C and over a wider range of cellular mutational profiles, unrestricted by the presence of BRAFV600E or wild-type PTEN. It attenuates xenografted tumor growth in immunocompromised mice to a degree comparable to I3C but at 1/10 th the dose. 1-benzyl I3C disrupts protein and transcript levels of the melanoma master regulator MITF-M and exogenous expression of MITF-M rescues the 1-benzyl I3C induced downregulation and antiproliferation. Western blots on cell extracts and immunofluorescence on xenografted tumors sections treated with 1-benzyl I3C show that unlike I3C, it inhibits MITF-M expression by downregulating β-catenin, which is unavailable in the nucleus to drive MITF-M transcription as confirmed by Chromatin Immunoprecipitation assay. 1-benzyl I3C significantly inhibited the promoter activity of wild-type MITF-M, which was rescued by mutating the LEF binding site on the promoter. Additionally 1-benzyl I3C inhibited TOP FLASH reporter activity confirming involvement of the Wnt/ β-catenin signaling cascade in its mechanism of action. Furthermore exogenous expression of constitutively active LRP6 rescued 1-benzyl I3C inhibition of TOP FLASH reporter activity implicating a direct binding target of 1-benzyl I3C functioning upstream of LRP6. Combination of 1-benzyl I3C and Vemurafenib showed cooperative anti-proliferative effect greater than each compound alone. Overall, this study introduces 1-benzyl I3C as a novel small molecule Wnt inhibitor with clinical potential, both as mono and combinatorial therapy for melanoma.

INTRODUCTION

Malignant melanoma, a melanocytic neoplasm of the skin accounts for less than 2% of all diagnosed skin cancers but 77% of all skin cancer related deaths [1]. Among the seven most common cancers in the US, melanoma is the only one whose incidence rate is on a continuous rise [2]. The increasing incidence, high mortality and high recurrence of malignant melanoma over the past decades pose an important clinical challenge for the development of novel therapeutics that can target the complexity of the underlying molecular signaling and effectively treat this disease with minimal adverse side effects and recurrence. Advances in high throughput sequencing technology have aided in genome profiling of these cells and provided insights into tumor heterogeneity, molecular features as well as the genetic diversity of melanomas. This has led to identification of some critical driver mutations like activating mutations in the proto-oncogenes BRAF and NRAS and inactivating mutations in the tumor suppressor proteins such as PTEN, TP53 and p16INK4a [3]. More than 60% of melanoma patients express a point mutation within a hot spot at position 600 on the BRAF gene. Approximately 90% of these mutations result in a T1799A transversion that substitutes Valine for Glutamate at residue 600 in the kinase domain of the protein, which leads to >200-500 fold hyperactivation of downstream pro-proliferative MEK/ERK signaling [4]. This oncogene addiction to BRAF for growth, survival and proliferation was effectively targeted by developing specific inhibitors of BRAF like Vemurafenib and Dabrafenib. Although initial clinical outcome was encouraging, showing dramatic attenuation of tumor burden, the long-term prognosis was poor due to development of acquired drug resistance.

A signature associated with increased Wnt signaling has recently been implicated to play a role in therapeutic escape [43]. Significantly elevated WNT5A expression was observed in subsets of tumors from patients exhibiting resistance to BRAF inhibitor therapy as well as in BRAF inhibitor resistant melanoma cell lines generated by long term in-vitro treatment with BRAF-inhibitor [5]. Cytoplasmic WNT5A was also reported to show increased expression with melanoma progression and correlated with poor outcome with BRAF inhibitor treatment [6]. Additionally very few treatment options are available for patients with non-BRAF mutant melanoma, which may also have hyperactivated Wnt signaling. Hence it is critical to design therapies for melanoma patients with BRAF mutation negative melanomas as well as patients who acquire drug resistance upon prolonged treatment with BRAF inhibitors. In this context targeting the Wnt/β-catenin signaling pathway seems to be a logical treatment strategy for melanoma.

However the role of Wnt signaling in melanoma remains controversial with literature replete with contradictory reports of Wnt exerting an oncogenic effect as well as Wnt activation correlating with improved prognosis and therapeutic benefit. A clinical study examined Wnt ligand expression in malignant melanoma and reported high levels different subtypes of Wnt correlating with the histopathological features of the tumor [7]. Melanomas characterised by small, uniform cells strongly expressed Wnt2, Wnt5a, Wnt7b, and Wnt10b. In contrast, melanomas characterized by large, pleomorphic cells expressed Wnt10b but did not express Wnt2 and had low levels of expression of Wnt5a. Expression of Wnt7b was variable in these

melanomas. In another study Wnt2 expression level was found to be high in melanoma cell lines, and antibodies against Wnt2 inhibited b-catenin signaling and suppressed tumor growth in an in vivo xenograft model by inducing apoptosis [8]

Most current studies implicate not Wnt proteins themselves but members of its downstream signaling cascade like b-catenin, axin and APC to be directly involved in tumorigenesis. In this context again the role of b-catenin in melanoma development is intriguing given that it too has demonstrated dual roles in both promoting and preventing melanoma progression. Supporting an activating role of β-catenin signaling in melanomagenesis, one study reported that constitutive activation of Wnt/b-catenin signaling enhances cell growth of murine melanoma cells in vitro [9]. Another group has shown that co-expression of stabilized nuclear β-catenin with oncogenic NRAS promotes melanoma formation in vivo and increased immortalization of melanocytes in vitro [10] In the same vein, a conditional mouse model of melanoma, based on melanocyte-specific PTEN loss and the BRAF V600E activating mutation, revealed a key role of β-catenin in mediating tumor progression and metastasis [11] In this PTEN/BRAF mouse model, increasing or decreasing b-catenin levels led to enhanced or repressed metastasis formation, respectively. Consistent with this, multiple immunohistochemistry studies of human melanomas revealed that many primary melanoma tumors have elevated levels of nuclear b-catenin [12]. Together these studies support an oncogenic role of Wnt in melanoma.

Interestingly recent studies have led to the emerging concept that in melanomas, MITF-M overexpression or amplification induces an increase in multivescicular bodies (MVB)/ late endolysozomes by vescicular traffic reprogramming. This in turn increases the sequestration of the "destruction complex" proteins like GSK3β and Axin in them, leading to enhanced canonical Wnt signaling. Wnt-driven stabilization of proteins (Wnt/STOP) is potentiated in these tumors, with many cellular proteins in addition to b-catenin becoming more stable. In melanomas, MITF itself is stabilized by increased expression triggered by nuclear b-catenin as well as increased stabilization by decreased C-terminal phosphorylation by GSK3β. This gives rise to a positive feedback loop between the melanoma master regulator MITF-M and an important proliferative signaling pathway like Wnt [13].

MITF-M has also been implicated in melanoma as a 'lineage survival' oncogene that is amplified in approximately 20% melanomas [14]. Overexpression of MITF-M, regulated epigenetically by transforming growth factor beta (TGF-β) has been shown to correlate with resistance to MEK inhibitor treatment [15]. Restoration of a MITF-driven transcriptional output either by genomic amplification or epigenetic means has also been identified as a mechanism for clinical resistance to BRAF inhibitors [16, 17, 18, 24].

Merging these concepts and findings, it is conceivable that a molecule that inhibits the Wnt signaling pathway can possibly downregulate MITF-M and essentially disrupt the positive feedback loop between these two critical players of melanomagenesis and drug resistance. Such a molecule can potentially not only delay or abrogate acquired drug resistance but can also be used to treat patients with BRAF mutation negative melanomas.

In our endeavor to find potent anti-cancer small molecules to treat melanoma, the potential of 1-benzyl I3C a synthetic analog of I3C was tested. The antiproliferative properties of 1-benzyl I3C

in reproductive cancer cells have been previously published from our laboratory and the formulation patented by our group [19]. 1-benzyl I3C displayed an approximate 1000-fold enhanced potency in suppressing the growth of estrogen-responsive as well as estrogen-independent human breast cancer cells [20]. The higher potency and greater efficacy of 1-benzyl I3C is based on its increased stability and lipophilicity as well as more efficient binding to its molecular target like human neutrophil elastase in human breast cancer cells. Both 1-benzyl-I3C and its parent compound I3C act as non-competitive allosteric inhibitors of elastase activity in these cells. In silico computational simulations identified a potential binding cluster on the external surface of the protease outside of the catalytic site. The Δ205 carboxyterminal truncation of elastase, which disrupts the predicted binding site, was enzymatically active and generated a novel 1-benzyl I3C resistant enzyme. Expression of the wild type and Δ205 elastase in MDA-MB-231 human breast cancer cells demonstrated that the carboxyterminal domain of elastase is required for the 1-benzyl-I3C inhibition of enzymatic activity, accumulation of the unprocessed form of the CD40 elastase substrate (a tumor necrosis factor receptor family member), disruption of NFκB nuclear localization and transcriptional activity, and induction of a G1 cell cycle arrest.

In the present study we demonstrate that 1-benzyl I3C shows >10fold potency than its parent compound I3C in causing antiproliferation both in melanoma cell lines in culture as well as in-vivo in melanoma xenografted immunocompromised mice. Unlike I3C or the clinically used oncogenic BRAF inhibitor Vemurafenib, 1-benzyl I3C is effective over a wider range of mutation profiles, unrestricted by the presence of oncogenic BRAF or wild-type PTEN. 1-benzyl I3C downregulates MITF-M expression like I3C, but via inhibition of signaling through the Wnt/β-catenin cascade. Additionally combinations of 1-benzyl I3C and Vemurafenib show a cooperative antiproliferative effect in multiple melanoma cell lines. Taken together this preclinical study identifies 1-benzyl I3C as a novel anticancer small molecule with translational potential both as a mono as well a combinatorial therapy to treat human melanoma.

MATERIALS AND METHODS

Cell Culture

Melanoma cell lines G-361, SK-MEL-28, SK-MEL-2, SK-MEL-30 and RPMI-7951 and melanocytes were purchased from American Type Culture Collection (ATCC) (Manasas, VA), and were authenticated according to the ATCC guidelines. DM738 melanoma cells were acquired from the tissue culture facility at University of California, Berkeley. The G361 melanoma cells were cultured in Modified McCoy's 5A cell media supplemented with 10% fetal bovine serum (Gemini Bio Products), 2 mM L- glutamine, and 2.5 ml of 10,000 U/ml penicillin/streptomycin mixture (Gibco, Life Technologies, Carlsbad, CA). DM738, SK-MEL-28 and SK-MEL-30 cells were cultured in DMEM with 4.5 g/L glucose and 2 mM L-glutamine supplemented with 10% fetal bovine serum, 2.5ml of 10,000 U/ml penicillin/streptomycin mixture in addition to 1X of MEM Non-Essential Amino Acid (Gibco, Life Technologies). RPMI-7951 and SK-MEL-2 melanoma cells were cultured in DMEM containing 4.5 g/L Glucose, 114 mg/L Sodium Pyruvate, and 2 mM L-glutamine, supplemented as described above. The cells were incubated in tissue culture dishes (Nalgene Nunc) at 37°C with controlled humidity and 5% CO2 air content.

Treatment with 1-benzyl Indole-3-carbinol, Indole-3-carbinol, Vemurafenib, Lithium Chloride (LiCl), 6-bromoindirubin-3′-oxime (BIO).

I3C and BIO was purchased from (Sigma Aldrich), Vemurafenib from (Adooq Bioscience), and LiCl from (Fischer Scientific). To study the effect of 1-benzyl I3C in comparison to its parent compound I3C and the commercial drug Vemurafenib, cells were treated with either 1,5, 10, 20, 25 μM of 1-benzyl I3C, 50, 100, 200, 300 μM I3C and 1, 5, 10, 15 μM Vemurafenib every 24 hours for 48hours and CCK-8 proliferation assay was performed on the cells at the end of the treatment. The same treatment regime was repeated on multiple melanoma cell lines with 20 μM 1-benzyl I3C and 200 μM I3C for 48hours to compare the relative sensitivities of the different cell lines with distinct mutation profiles to 1-benzyl I3C and I3C. Cells were treated with 20 μM 1-benzyl I3C every 24hours for 72 hours and harvested at 24, 48 and 72 hours for western blot and flow cytometric analysis. Cells were treated with 20 μM LiCL or 10 μM BIO for 48 hours before harvesting them for western blots. For the experiments on the combination of 1-benzyl I3C and Vemurafenib treatment, cells were treated with the compounds for 24 hours. I3C, Vemurafenib, LiCL and BIO were dissolved in 99.9% HPLC grade DMSO (Sigma Aldrich, Milwaukee, WI) and the final dilution was performed in the media aliquots used for treatment.

MTT proliferation assay

Melanoma cell lines were seeded on a 48well plate and upon 80-90% confluency were either treated in triplicates with different concentrations of 1-benzyl I3C, I3C or Vemurafenib as well

as combinations of 1-benzyl I3C and Vemurafenib or DMSO for time durations specified for each experiment. Subsequently inhibition of proliferation was assessed using Cell counting Kit - 8 (Dojindo) as per the protocol in the user's manual. Briefly, 50µl of the CCK-8 solution was added to each well along with 450 µl media and incubated for 2-3 hours. The absorbance was read at 450nm and % inhibition was calculated for each condition standardizing DMSO to zero using the formula: [(100 – Value of treatment/ Value of DMSO treated control) X100]

Flow cytometry analysis of DNA content

Melanoma cells were treated with the indicated concentrations of 1-benzyl I3C in triplicates every 24 hours and harvested at the end of 24, 48 or 72 hours for cell cycle analysis. The DNA content of propidium iodide stained nuclei from harvested cells were determined by flow cytometry. Briefly, cells were hypotonically lysed in 300 mL of DNA staining solution (0.5 mg/mL propidium iodide, 0.1 % sodium citrate, and 0.05 % Triton-X 100). Emitted fluorescence from the nuclear of wavelengths more than 585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Ten thousand nuclei were analyzed from each sample at a rate of 300-500 nuclei/second. The percentage of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems in the Cancer Research Laboratory Microchemical Facility of the University of California, Berkeley.

Western blot Analysis

Western Blot analyses of samples electrophoretically fractionated on 12% acrylamide gels were carried out as previously described (18, 19). ECL Lightening reagents were used to visualize the primary antibody bound protein bands in nitrocellulose membranes and the results captured on ECL Autoradiography Film (GE Healthcare, Piscataway, NJ). The western blots employed the following primary antibodies: mouse anti-MITF-M (Thermo-scientific), mouse anti-HSP 90 (BD Biosciences), mouse anti-CDK2, rabbit anti-CDK4, rabbit anti-CDK-6, mouse anti-rabbit BRAF, goat anti-LEF-1, rabbit anti-AXIN, mouse anti-BCL2, (Santa Cruz), rabbit anti-P21, mouse anti-Cyclin D1, rabbit anti-MEK-p, rabbit anti-MEK1/2, rabbit anti-GSK3β rabbit anti-LRP6, rabbit anti-LRP6-phospho (Cell signaling), mouse anti-β-catenin (Invitrogen).

RT-PCR analysis

Total RNA was extracted from harvested cells using the RNeasy extraction kit for mammalian cells (obtained from Qiagen, Hercules, CA) and spectrophotometrically quantified by absorbance at 260 nm. Reverse transcription (RT) reactions were carried out using RT-MMLV reverse transcriptase (Invitrogen, Eugene, OR) and the cDNA was used for PCR reactions using 10 pM of the following primers: MITF-M forward 5' CCG TCT CTC ACT GGA TTG GT 3', MITF-M Reverse 5' TAC TTG GTG GGG TTT TCG AG 3' GAPDH forward, TGAACGGGAAGCTCACTGG and reverse, TCCACCACCCTGTTGCTGTA. PCR conditions

were as follows: 30s at 94 ^{o}C, 30s at 55 ^{o}C and 30s at 72 ^{o}C for 28 cycles. PCR products were electrophoretically fractionated in 1.5 % agarose gels containing 0.01% Gel Red (Biotium) for DNA staining along with 1kb plus DNA ladder (Thermo Scientific) and further visualized by a UV transilluminator.

Tumor Xenografts and Immunofluorescence

Approximately 1 million G361 melanoma cells in a total volume of 0.1 ml of Matrigel were injected subcutaneously into each lateral flank of NIH III athymic nude mice. The resulting tumors were allowed to grow to a mean starting volume of 146 ± 10 mm^3. The animals were then randomized into two groups; a vehicle control group that was treated with DMSO and an I3C treated group that were injected daily with 200mg/kg body weight of I3C. The resulting tumor volumes were measured every other day for four weeks using calipers. The tumor volumes were calculated using the standard formula: (Width^2 X Length)/2, and changes in tumor volumes were calculated using the formula: 100+{(Tf-Ti)/ Ti X100}, where Tf is the final mean tumor volume and Ti is the initial mean tumor volume. At terminal sacrifice, tumor xenografts were harvested, and a portion of each tumor was fixed in 4% para-formaldehyde for 1 hour at room temperature, followed by a PBS wash and subsequently immersed in 3% sucrose overnight at 4°C. The resulting tissues were embedded in OCT and 10 μm thin sections were taken for immunofluorescence studies.

Luciferase reporter assay.

G361 Melanoma cells were cultured to approximately 80% confluency in 6 well adherent culture plates in triplicates for each experimental condition for 24 hours. The cells were then transiently transfected with either a wild-type pGL2-pMITF -333/+120- luciferase reporter plasmid or a similar plasmid with the LEF-1/TCF binding site mutated at – 199. pGL2-pMITF-333/+120-ΔLEF-1-luciferase, (ΔLEF-1) was generated using the following primers:

LEF-1 forward, 5'- GACAGTGAGTTTGACTTTGGCAGCTCGTCACTTAA -3'

LEF-1 reverse, 5'-TTAAGTGACGAGCTGCCAAAGTCAAACTCACTGTC -3'.

PCR conditions used were as follows: 30s hotstart at 95°C, 30s at 95°C, 1min at 55°C, 8 min at 68°C for 16cycles. 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). SuperFect Transfection Reagent (Qiagen) was used for the transfections as per instructions in the user manual. The next day the cells were either treated with 200μM I3C or an equal volume of the DMSO vehicle control for both wild-type plasmid transfected as well as mutant plasmid transfected cells. 24 hours later luciferase assays were performed by harvesting cells in 1X Promega Lysis Buffer, allowing it to incubate for 15

minutes at room temperature, followed by 15minutes incubation on ice. The cells were then pelleted by a spin at 15,000 rpm for 1 minute at 4°C. 20µl of supernatant from each sample was combined with 100µl luciferase substrate (Promega) and emitted fluorescence was measured using luminometer, Lumat LB 9507 (EG&G Berthold) and the relative light units (RLU) were recorded. The RLU/µg for each sample was calculated by dividing the data obtained with their specific protein concentration determined by a Bradford assay using 1X BioRad Protein Assay reagent and measuring O.D at 595nm.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed using G361 melanoma cells treated with or without 200 µM I3C for 72 hours. Proteins were crosslinked to DNA by the addition of formaldehyde at 2.5% of the final concentration for 5 minutes at room temperature to cultured cells. Fixation was quenched with glycine for a final concentration of 125 mmol/l for 5 minutes. Harvested cells were sonicated and the chromatin was immunoprecipitated overnight and 15µl of mouse anti-Brn2 antibodies (Santa Cruz Biotechnology) was added to the cell lysates to immunoprecipitate the chromatin-bound Brn2 protein. A fragment of the MITF-M promoter (-170 +120) containing the BRN2 binding site (-53 to -27) was amplified using the primers- MITF-M Forward: 5'- CGT CAC TTA AAA AGG TAC CTT TAT ATT TAT G-3' MITF-M Reverse: 5' –TGT TTTAGC TAG CAC CAA TCC AGT GAG AGA CGG-3' by cycling 36 times (95^{O}C, 30 s/52^{O}C 30 s/72°C, 30 s) with a 72°C, 10 min extension. The PCR products were electrophoretically fractionated on a 1.5% agarose gel and visualized using a transilluminator.

Wnt conditioned media

Murine fibroblast L cells stably transfected with a construct expressing high levels of humanWnt3A and its corresponding empty vector transfected control cells was obtained as a kind gift from Dr. Henk Roelink. The cells were grown in DMEM containing 4.5 g/L Glucose, 114 mg/L Sodium Pyruvate, and 2 mM L-glutamine, supplemented with with 10% fetal bovine serum (Gemini Bio Products), 2 mM L- glutamine, and 2.5 ml of 10,000 U/ml penicillin/streptomycin mixture (Gibco, Life Technologies). Conditioned media was harvested in two batches, once during routine media change after 2 days of seeding the cells and once more when the plates were confluent. The cells were trypsinized as described earlier using .025% trypsin (Lonza) and pelleting the cells by a spin at 4000rpm for 5 minutes and aspirating and filtering the supernatant through a .2micron filter to obtain the conditioned media. The two batches of conditioned media were mixed 1:1 and stored in aliquots at -80°C for future use.

TOP FLASH Reporter Assay

The TOPflash/FOPflash (Millipore) reporter assay was performed on G361 cells to assess the effect of 1-benzyl I3C on β-catenin driven Wnt transcriptional activity. TOPflash is a TCF/LEF

responsive luciferase construct encoding the firefly luciferase reporter under the control of a minimal (m) CMV promoter and tandem repeats of a TCF/LEF transcriptional response element (TRE). FOPflash is a similar construct with the LEF-1 binding site mutated which serves as the negative control. G361 cells were cultured to approximately 80% confluency in a 6 well clear bottom plate (Nunc). The TOPflash/FOPflash construct was transfected using Superfect Transfection Reagent (Qiagen.) according to the manufactures instructions. Cells in each well were transfected with 2µg DNA and 4ul Superfect for 2-3 hours and then replaced with 2ml of 1:1 ratio of fresh and conditioned media from cultured L-cells secreting Wnt or no Wnt. 24 hours later the cells were treated with/without 10µM 1-benzyl I3C for another 24 hours in conditioned media. Cells were washed with DPBS, resuspended in Lysis Buffer (Promega), incubated on ice for 15minutes, spun at 14000rpm for 1 minute at 4°C. and the supernatant is used for detection of luciferase activity. 20µl of supernatant from each sample was combined with 100µl luciferase substrate (Promega) and emitted fluorescence was measured using luminometer, Lumat LB 9507 (EG&G Berthold) and the relative light units (RLU) were recorded. The RLU/µg for each sample was calculated by dividing the data obtained with their specific protein concentration determined by a Bradford assay using 1X BioRad Protein Assay reagent and measuring O.D at 595nm. The experiment was performed in triplicates at least 3 times.

RESULTS

1-benzyl I3C causes antiproliferation in melanoma cell culture and in-vivo at >10-fold lower concentration than I3C and shows sensitivity over a wider range of mutation profiles.

To test if 1-benzyl I3C showed antiproliferative effects in melanoma comparable to its parent compound I3C, two I3C sensitive cell lines, G-361 and DM 738 were treated with varying doses of 1-benzyl I3C for 48hours and cell proliferation was determined using CCK-8 assay. 1-benzyl I3C not only showed significantly greater antiproliferation than I3C but also at 10-20μM concentration that is 10-20fold lower than the optimum concentrations of I3C at 200-300 μM. Further these concentrations of 1-benzyl I3C were similar to the optimum concentrations of the clinically used BRAF inhibitor Vemurafenib. Interestingly unlike I3C and Vemurafenib, which are ineffective in melanoma cell lines expressing wild-type BRAF such as SK-MEL-2, 1-benzyl I3C showed antiproliferation in these cells as well. This indicated that 1-benzyl I3C possibly has a different mechanism of action that was independent of the presence of oncogenic BRAF **[Fig 1A]**.

So we wanted to test if there was any mutational determinant to predict sensitivity to 1-benzyl I3C. We treated 5 different melanoma cells lines with mutation profiles encompassing some of the most common mutations associated with melanoma namely BRAF, PTEN, N-RAS and P53 and treated them with the optimum dose of 20μM 1-benzyl I3C and 200μM I3C for 48 hours. 1-benzyl I3C showed a much wider range of sensitivity than I3C and Vemurafenib being effective in cell lines harboring wild-type BRAF as well **[Fig 1B]**. However no such antiproliferative response was observed upon treatment of normal epidermal melanocytes with 1-benzyl I3C indicating that it specifically targets melanoma cells and therefore has translatable potential.

Hence we extended this study to an in-vivo murine model of melanoma generated by injecting G-361 cells into the flanks of athymic nude immunocompromised mice to generate palpable xenografted tumors. The mice were subcutaneously injected with the optimum dose of 20mg/kg body weight 1-benzyl I3C everyday, for a 4-week time course and the size of the tumors were measured every other day and volumes calculated as described in the methods. The treatment could be extended beyond a month since the vehicle treated tumor size was beyond the permitted size for humane animal treatment for research. 1-benzyl I3C strongly attenuated tumor growth compared to the vehicle control that was noticeable within the first week of injections and sustained over the entire time course **[Fig 1C]**. At termination of treatment, the mice were sacrificed and the residual tumors were harvested. The size of the residual tumors treated with 1-benzyl I3C was significantly smaller than the vehicle control and comparable to the tumors treated with 10 fold higher concentration of I3C **[Fig 1C right panel]**. This confirmed that like in breast cancer, 1-benzyl I3C is a significantly more potent molecule than I3C even against melanoma.

1-benzyl I3C downregulates key cell-cycle regulators to cause a G1phase arrest in sensitive cells.

To understand the molecular mechanism underlying this antitumor effect, flow cytometric analysis was performed on multiple melanoma cell lines as well as normal melanocytes, treated with varying doses of 1-benzyl Indole-3-carbinol for 48hours. A significant G1 cell cycle arrest was observed in all the melanoma cell lines with no comparable effect on normal melanocytes, implying that 1-benzyl Indole-3-carbinol selectively affects melanoma cells by inducing a G1 cell cycle arrest **[Fig 2A, B, C, D, E, F left panel].** Next similar flow cytometric analysis was performed on the cell lines with the optimum dose of 20μM 1-benzyl I3C for a time course of 24, 48, 72hours to examine the temporal profile of its antiproliferative effect **[Fig 2A, B, C, D, E, F Right Panel].** Western blot analyses performed on cell lines treated with 1-benzyl I3C for 24, 48, 72 hours revealed downregulation of key cell cycle regulators like CDK 2, 4, 6, concomitant to the G1 cell cycle arrest induced by 1-benzyl Indole-3-carbinol **[Fig 2G].**

Inhibition of MITF-M expression by 1-benzyl I3C is necessary for induction of its antiproliferative effect.

Since the 1-benzyl I3C inhibited cell cycle regulators are down stream targets of the melanoma master regulator– MITF-M, it was logical to examine if it was affected by 1-benzyl I3C treatment as well. Western blots on G361 and DM738 cells treated with 1-benzyl I3C for 24, 48, 72 hours showed a significant downregulation of MITF-M starting as early as 24 hours similar to observations with I3C treatment. Interestingly the WT BRAF expressing SK-MEL-2 cells which were not sensitive to I3C or Vemurafenib and showed no downregulation of MITF-M levels upon treatment with I3C, shows a significant inhibitory effect on MITF-M protein levels with 1-benzyl I3C treatment **[Fig 3A]**. RT PCR performed on these cells post 1-benzyl I3C treatment showed a concomitant inhibition of MITF-M transcript level accounting for the observed downregulation of MITF-M protein **[Fig 3B]**.

To further determine if MITF-M is necessary to mediate 1-benzyl Indole-3-carbinol's antiproliferative effect, we overexpressed MITF-M in sensitive G361 cells by transiently transfecting it with plasmid expressing MITF-M (pCMV-MITF). Subsequently the cells were treated with or without 1-benzyl I3C for 24 hours and proliferation was determined using a CCK-8 assay. Overexpressing MITF-M not only rescued 1-benzyl Indole-3-carbinol's antiproliferative effect, but also the downregulation on cell cycle regulator genes like CDK4 as well as pro-apoptotic genes like BCL2 as determined by western blots **[Fig 3C,D]**.

1-benzyl I3C disrupts signaling through the Wnt/ β-catenin pathway.

We wanted to determine the molecular mechanism by which 1-benzyl Indole-3-carbinol downregulated levels of MITF-M transcripts. The observation that the antitumor effect of the compound is not restricted to the BRAF V600E expressing melanoma cell lines, we examined its

effect on other signaling pathways known to regulate MITF-M. Since one-third of human melanomas specimens show activation of the canonical Wnt pathway evident from the presence of nuclear β-catenin, we tested if 1-benzyl I3C treatment affected the Wnt/β-catenin pathway [21]. Additionally, recent studies have implicated β-catenin signaling in a key role in melanoma progression, tumor cell survival and chemoresistance [22]. From these evidence we hypothesized that a compound that downregulates β-catenin can potentially inhibit MITF-M expression to induce apoptosis in metastatic melanoma and will be effective against a wider range of melanoma phenotypes, since it would function independent of the presence of the oncogenic BRAF and 1-benzyl I3C seemed to fit the bill.

Western blots performed on melanoma cell lines treated with 1-benzyl I3C revealed that different components of the Wnt/β-catenin pathway were indeed significantly affected by the treatment. β-catenin protein levels were significantly downregulated in all the cell-lines, which could be explained by the observed upregulation of components of its destruction complex such as GSK3β and Axin, that function to restrict β-catenin to the cytoplasm and phosphorylates it for inactivation and subsequent degradation in absence of the growth factor Wnt. β-catenin therefore cannot enter the nucleus to perform its function of regulating the transcription of its target genes like MITF-M. Additionally the Wnt co-receptor LRP5/6 itself was significantly downregulated implicating that 1-benzyl I3C possibly binds directly to LRP5/6 or to some component of the Wnt signaling pathway functioning upstream of it **[Fig 4A].** The effect of 1-benzyl I3C on the Wnt signaling pathway was further confirmed by immunofluorescence on sections taken from 1-benzyl I3C treated tumor xenografts, which mimicked the cell culture data, thereby reiterating the involvement of this signaling cascade in 1-benzyl I3C's anti-tumor effect **[Fig 4B].**

β-catenin functions at the node of multiple signal transduction pathways and is stabilized by Wnt independent mechanisms as well [5,6]. Hence to confirm the involvement of the canonical Wnt signaling in 1-benzyl I3C's mechanism of action, TOP FLASH assays were performed. G361 cells were cultured in conditioned media harvested from either mouse fibroblast cells (Lcells) stably transfected with empty CMV vector or L-cells expressing pCMV-Wnt. It is presumed that the conditioned media from the Wnt expressing cells will contain large amounts of the secreted growth factor and hence will be able to trigger Wnt signaling in the G361 cells. The G361 cells were transiently transfected with either a multiple LEF binding site containing enhancer driven minimal promoter luciferase reporter construct (TOP) or a similar construct in with mutant LEF binding site to serve a negative control (FOP). 24hours post transfection, the TOP as well as FOP transfected cells were treated either with DMSO or 1Benzyl-Indole-3-carbinol for 24 hours and the luciferase activity was measured. In the cells growing in the Wnt containing conditioned media, the DMSO vehicle control treated cells showed massive Wnt signaling detected by very high luciferase activity. However upon treatment with 1-benzyl Indole-3-carbinol, Wnt signaling was significantly attenuated evident from the loss of luciferase activity thereby confirming the involvement of the canonical Wnt signaling cascade in 1-benzyl Indole-3-carbinol's mechanism of action **[Fig 5A]**.

Using *in-silico* modeling to predict potential 1-benzyl Indole-3-carbinol binding site on different signaling component of the Wnt pathway indicated a potential direct binding site of our molecule on the growth factor Wnt itself **[Fig 5B]** and not on its receptor Frizzled or the co-receptor LRP5/6. To indirectly test the veracity of this prediction TOP FLASH assays were performed

like before but this time the cells were additionally co-transfected with either an empty CMV vector or a vector expressing constitutively active LRP6 (pCMV-LRP), which acts a co-receptor for Wnt with its direct binding receptor Frizzled. In the wild-type vector transfected cells, treatment with 1-benzyl I3C significantly inhibited the Wnt signaling compared to the vehicle treated control. However in the constitutively active LRP6 transfected cells, 1-benzyl I3C induced inhibition of luciferase activity was significantly rescued **[Fig 5C]**. This shows that 1-benzyl I3C has a direct binding target upstream of LRP5/6, reiterating the possibility that 1-benzyl I3C could be directly binding to the Wnt ligand itself to disrupt its binding to Frizzled or disrupt formation of the receptor complex with Frizzled and LRP6, resulting in downregulation of the signaling cascade through GSK3β/β-Catenin/LEF/MITF-M.

β-catenin mediates 1-benzyl I3C's regulation of MITF-M transcript expression and antiproliferation

We wanted to experimentally test if the MITF-M transcript inhibition by 1-benzyl I3C is a direct result to the observed downregulation of Wnt signaling. We took a pharmacological approach to confirm the role of β-catenin in the transcriptional downregulation of MITF-M induced by 1-benzyl I3C. 1-benzyl I3C sensitive melanoma cells, G361, SK-MEL-2, were pre-treated with inhibitors of the β-catenin inhibitor-
GSK3β, like Lithium Choride (LiCl) or 6-bromoindirubin-3'-oxime (BIO), which is a potent reversible ATP-competitive inhibitor of GSK3β. The treatment is continued for 3 hours prior to treatment with or without 1-benzyl I3C for 24hours. Western blots on these cells demonstrated a significant rescue of the 1-benzyl Indole-3-carbinol induced downregulation of β-catenin, as well as its transcriptional target MITF-M and its downstream cell cycle regulator target like CDK2 and anti-apoptotic target like BCl2 upon treatment with the GSK3β inhibitors **[Fig 6A].** This implicated a critical role of β-catenin mediated regulation of MITF-M in 1-benzyl Indole-3-carbinol's antiproliferative effect.

β-catenin is a known transcriptional regulator of MITF-M that interacts with the co-factor TCF/LEF to bind to the putative LEF binding site on the MITF-M promoter at (-199 to -193 CTTTGAT). To further elucidate the direct outcome of downregulated β-catenin on MITF-M transcriptional activity Chromatin immunoprecipitation (ChIP) assay was performed on G361 cells treated with or without 1-benzyl I3C for 48 hours. Harvested cells were sonicated to shear the genomic DNA, which was subsequently cross-linked to protein and immunoprecipitated with either anti-LEF1 or with an IgG control antibodies. PCR analysis using primers specific to the LEF1 binding site in the MITF-M promoter, revealed that 1-benzyl I3C significantly disrupted nuclear LEF1 interactions with this promoter. One percent input was used as a loading control **[Fig 6B].**

Next, to determine if the decreased binding of LEF-1 to the MITF-M promoter affects promoter activity and accounts for the observed reduction in MITF-M transcript levels, G361 melanoma cells were transiently transfected with a wild-type -333/120 MITF-M promoter- luciferase reporter plasmid (WT) and luciferase activity was determined post treatment with or without 1-benzyl I3C for 24hours. Luciferase activity was significantly abrogated in the 1-benzyl I3C treated cells compared to the DMSO vehicle treated control, confirming the inhibitory effect of

1-benzyl I3C on the MITF promoter activity. Mutation of the LEF1 consensus site (LEF1 Mut), prevented this 1-benzyl I3C down regulation of MITF-M promoter activity, directly implicating the involvement of LEF-1 in the inhibition of MITF-M promoter activity by 1-benzyl I3C **[Fig 6C].**

1-benzyl I3C and Vemurafenib exhibit a cooperative antiproliferative effect in melanoma cells.

Since Vemurafenib selectively targets the oncogenic BRAF pathway and 1-Benzyl Indole-3-carbinol targets the Wnt signaling pathway and both the pathways play a significant role in melanomagenesis and progression, we hypothesized that a combination of the two compounds will synergize to cause greater antiproliferative effect that each compound alone. CCK-8 proliferation assays performed using various combinations of 1-benzyl I3C and Vemurafenib indeed showed a co-operative antiproliferative effect **[Fig 7A].**

Western blots were performed to examine the effects of combinations of 20μM 1-benzyl I3C and 10 μM Vemurafenib on MITF-M as well as the BRAF V600E and Wnt signaling was examined in G-361, DM738 and SK-MEL-2 melanoma cells, after 24 hours treatment with 1-benzyl I3C. In G361 and DM738 cells the combination of 1-benzyl I3C and Vemurafenib cooperatively down-regulated MITF-M protein levels, by inhibiting both oncogenic BRAF signaling evident from downregulated MEK-P with Vemurafenib treatment and inhibiting Wnt signaling evident from upregulated GSK3β and downregulated β-catenin protein levels with 1-benzyl I3C treatment. In SK-MEL-2 cells the inhibition on MITF-M was primarily by inhibition of Wnt signaling since the expression of wild-type BRAF rendered it not sensitive to Vemurafenib action **[Fig 7B].** Overall, the combinational inhibitory effects of I3C and Vemurafenib on melanoma cell proliferation mirrored the down regulation of MITF-M levels. Hence this novel combination of a BRAF inhibitor and a Wnt Inhibitor, which has never been reported till date to treat melanoma, could prove to be a more lethal combination of drugs, usable in the clinic, than other combinations of commercially available drugs that target different components of the same BRAF signaling pathway and potentially delay or perhaps abrogate the emergence of acquired drug resistance.

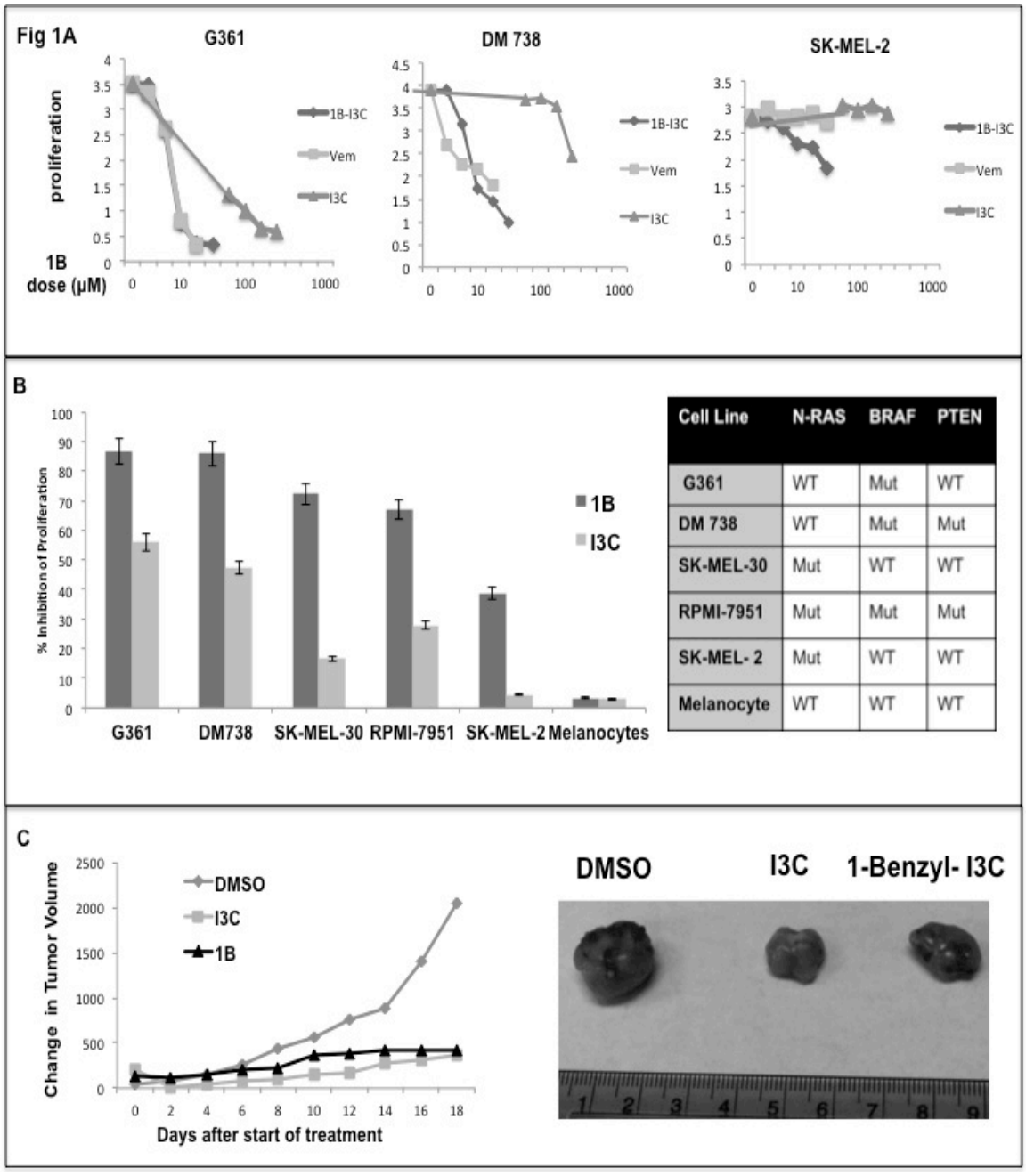


Fig 1A
G361
DM 738
SK-MEL-2
proliferation
1B dose (μM)
1B-I3C
Vem
I3C
B
% Inhibition of Proliferation
1B
I3C
G361
DM738
SK-MEL-30
RPMI-7951
SK-MEL-2
Melanocytes
Cell Line | N-RAS | BRAF | PTEN
G361 | WT | Mut | WT
DM 738 | WT | Mut | Mut
SK-MEL-30 | Mut | WT | WT
RPMI-7951 | Mut | Mut | Mut
SK-MEL- 2 | Mut | WT | WT
Melanocyte | WT | WT | WT
C
Change in Tumor Volume
DMSO
I3C
1B
Days after start of treatment
DMSO
I3C
1-Benzyl- I3C

Figure 1.

Potency of 1-benzyl I3C against human melanoma in cell culture and vivo in melanoma tumor xenografts.

A. Oncogenic BRAF expressing and I3C, Vemurafenib sensitive G361 and DM738 cells as well as Wild type BRAF expressing and I3C, Vemurafenib resistant SK-MEL-2 cells were treated with 20μM 1-benzyl I3C. Its effect on proliferation of these cells was determined using a CCK-8 cell proliferation assay.

B. Human melanoma cell lines with distinct genotypes were treated with or without 20μM 1-benzyl I3C for 48 hours and the effects on cell proliferation measured using a CCK-8 assay relative to the vehicle control.

C. Immunocompromised athymic nude mice with G-361 cell-derived xenografted tumors were subcutaneously injected with either 1-benzyl I3C or with DMSO vehicle control, and resulting tumor volumes were calculated as described in the Material and Method section. The micrograph insert shows tumors harvested at week 4.

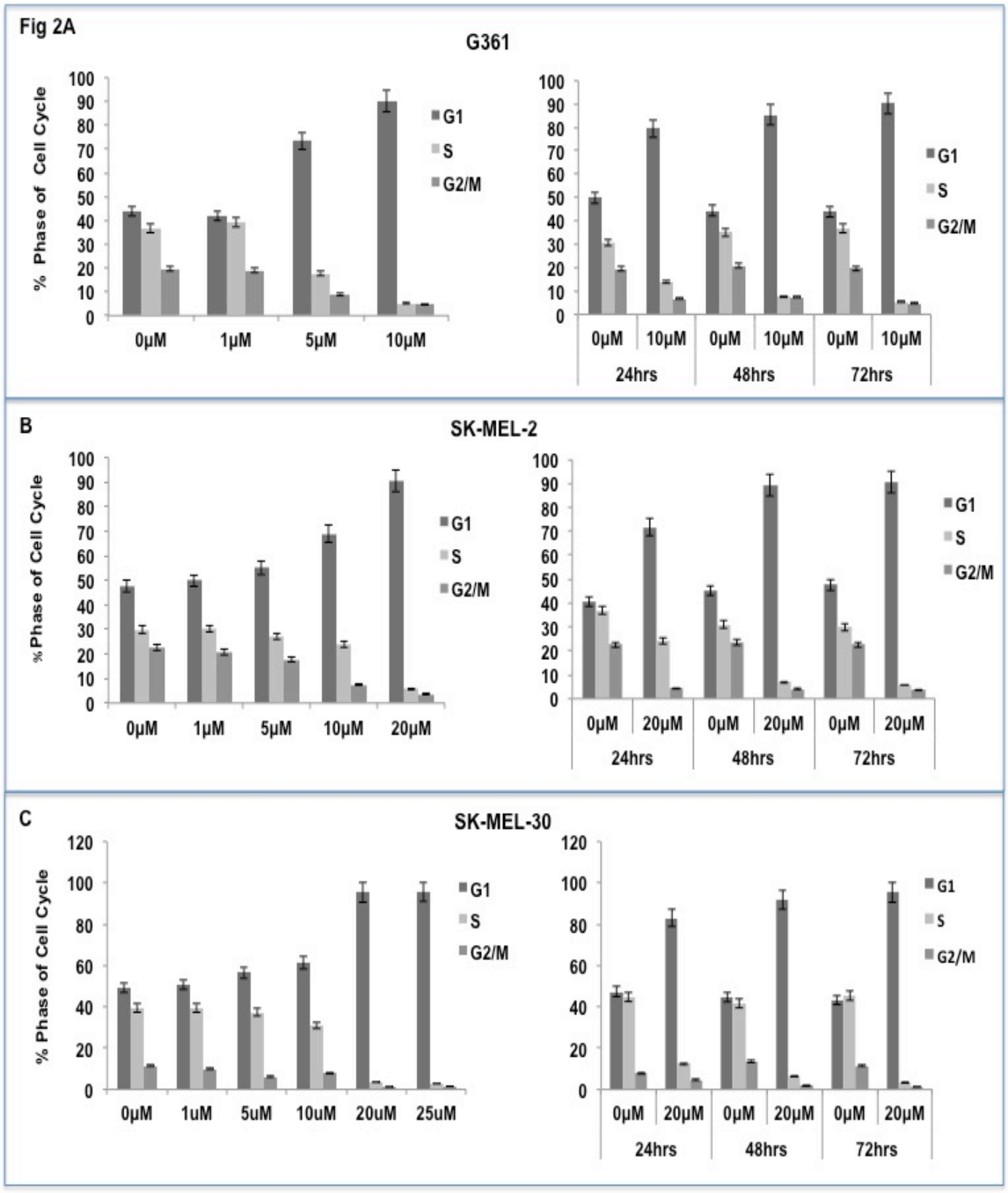


Fig 2A
G361
% Phase of Cell Cycle
100
90
80
70
60
50
40
30
20
10
0
G1
S
G2/M
0μM
1μM
5μM
10μM
0μM
10μM
0μM
10μM
0μM
10μM
24hrs
48hrs
72hrs
B
SK-MEL-2
% Phase of Cell Cycle
0μM
1μM
5μM
10μM
20μM
0μM
20μM
0μM
20μM
0μM
20μM
24hrs
48hrs
72hrs
C
SK-MEL-30
% Phase of Cell Cycle
120
100
80
60
40
20
0
0μM
1uM
5uM
10uM
20uM
25uM
0μM
20μM
0μM
20μM
0μM
20μM
24hrs
48hrs
72hrs

Figure 2.

Dose dependent and temporal effects of 1-benzyl I3C on the cell cycle in melanoma cell lines with a range of mutation profiles.

Human melanoma cells namely G361, DM738 and SK-MEL28 **(A,D,E)** expressing oncogenic BRAF-V600E and wild type BRAF expressing SK-MEL-2 and SK-MEL-30 **(B,C)** cells as well as normal epidermal melanocytes were treated with the indicated concentrations of 1-benzyl I3C for indicated time durations. Harvested cells were stained with propidium iodide, and the DNA content of stained nuclei were quantified by flow cytometric analysis as described in the Materials and Method section. The histograms of representative experiments from three independent trials are shown and the percentage of cells in the population displaying G1, S or G2/M DNA content was quantified.

In Figure 2 the Left panels show that 1-benzyl I3C induced a dose-dependent G1 cell cycle arrest in all of the cell lines examined and the response, unlike I3C was independent of presence of oncogenic BRAF-V600E or PTEN. The right panel shows the 24, 48 and 72 hours treatment response profile of the same cell lines. Evidently in all the cell lines 1-benzyl I3C induces a significant G1 cell cycle arrest as early as within 24hours of treatment and the effect persists through 48 to 72hours. In case of cell lines like G361, SK-MEL-2 and SK-MEL-30, the G1 arrest accentuates with time. Additionally 1-benzyl I3C did not show any such G1 phase cell cycle arrest in the normal epidermal melanocytes accounting for the resistance of these cells to the anti-proliferative effect of 1-benzyl I3C as observed in the CCK-8 proliferation assay

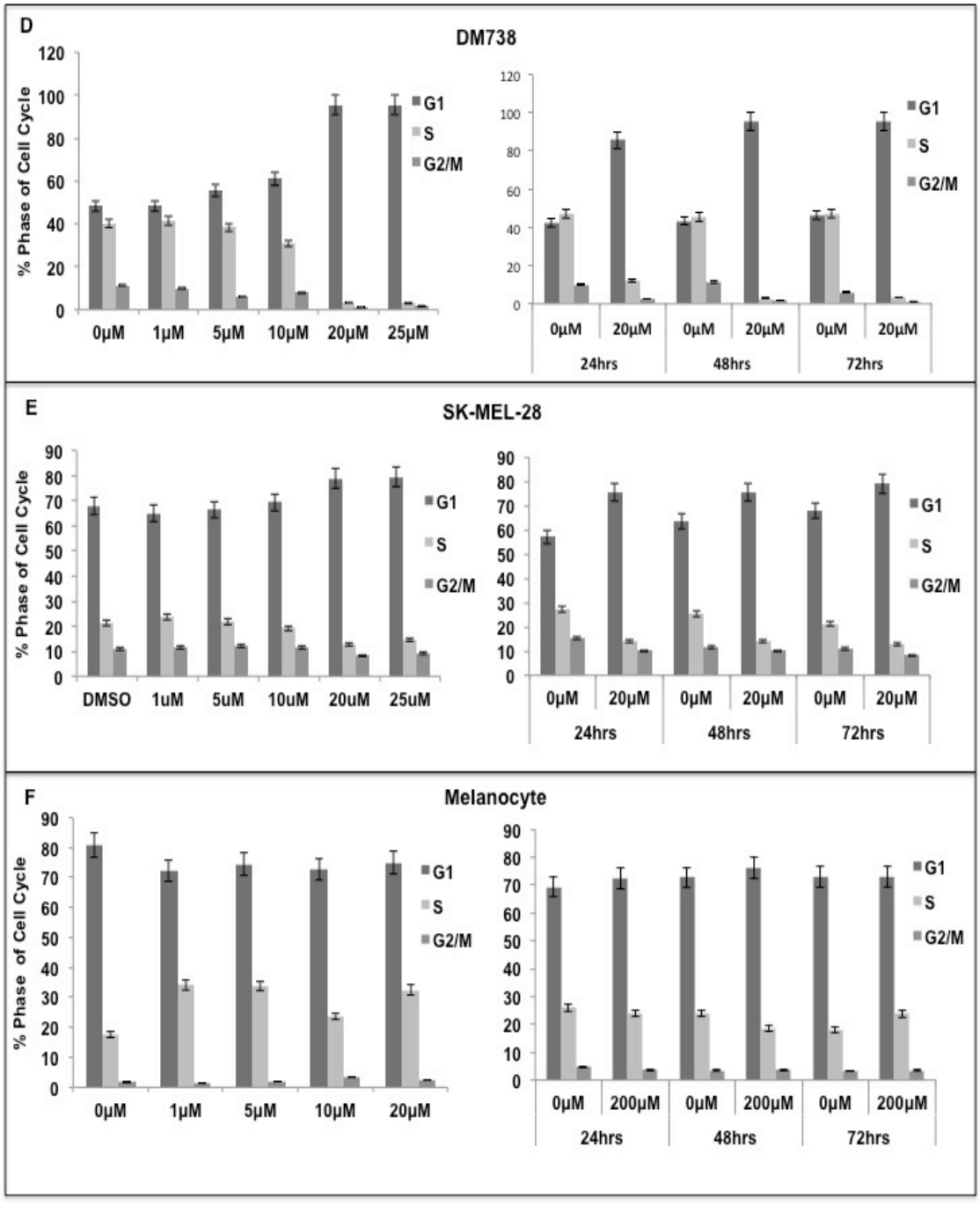

D
DM738
% Phase of Cell Cycle
G1
S
G2/M
0μM
1μM
5μM
10μM
20μM
25μM
24hrs
48hrs
72hrs
E
SK-MEL-28
DMSO
1uM
5uM
10uM
20uM
25uM
F
Melanocyte
200μM

Figure 2G.

Effects of 1-benzyl I3C treatment on key cell cycle regulators over a time course in human melanoma cells.

Cultured human melanoma cell lines that express oncogenic mutant BRAF V600E alongside wild type PTEN (G361) or mutant PTEN (DM738) or wild type BRAF and wild type PTEN expressing (SK- MEL-2) cells were treated with or without 20μM I3C over a 72hours time course.

Total cell lysates were fractionated by SDS-polyacrylamide electrophoresis and the levels cell cycle regulators like CDK2, CDK4, Cyclin D1 and p21 were determined by western blot analysis in comparison to the HSP90 gel loading control. Representative blots from three independent experiments are shown.

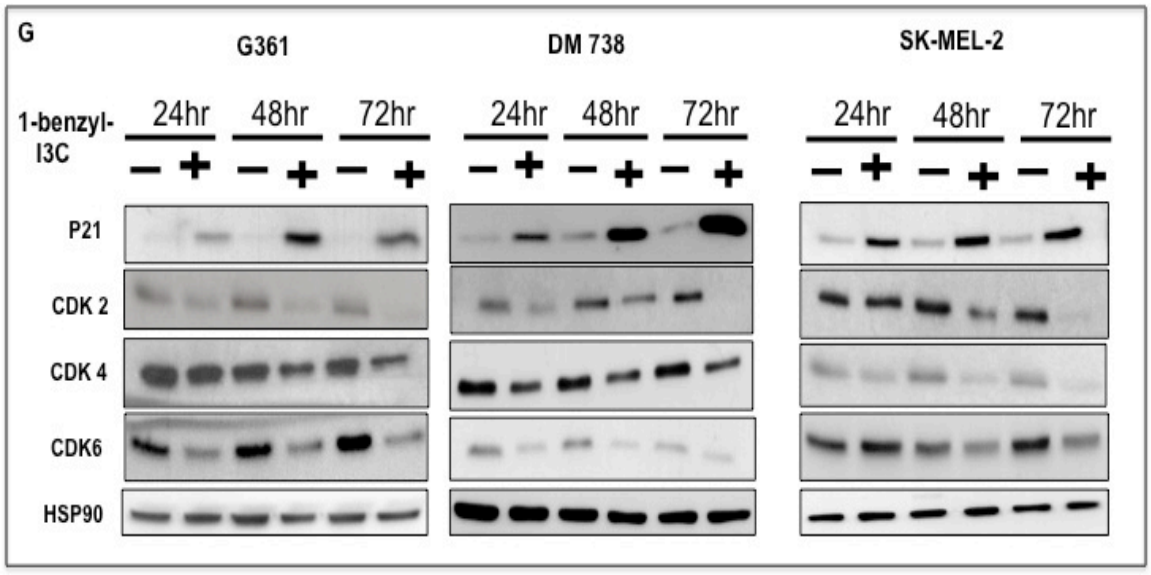

G
G361
DM 738
SK-MEL-2
1-benzyl-I3C
24hr
48hr
72hr
P21
CDK 2
CDK 4
CDK6
HSP90

Figure 3.

1-benzyl I3C regulation of MITF-M expression to mediate its anti-proliferative effect in melanoma.

A. The levels of MITF-M protein were determined in melanoma cells treated with 10μM concentration of 1-benzyl I3C for a time course of 24, 48 and 72 hours by western blots.

B. The effect of 1-benzyl I3C on MITF-M transcript expression in the same cells as Fig 3A namely G-361, DM738, SK-MEL-2, treated with or without 20μM I3C was determined by RT-PCR analysis in comparison to the GAPDH control.

C. G-361 cells were either transfected with pCMV-MITF expression vector, or pCMV empty vector control or left untransfected, and each set of cells were treated with or without 20 μM I3C for a submaximal time of 24 hours. Cell proliferation was measured using a CCK-8 assay, and results show the mean of three independent experiments ± SEM (*, $p < 0.01$)

D. Levels of MITF-M and its downstream targets - anti-apoptotic BCL2 and pro-proliferative CDK4 protein levels were determined in comparison to HSP90 by western blots.

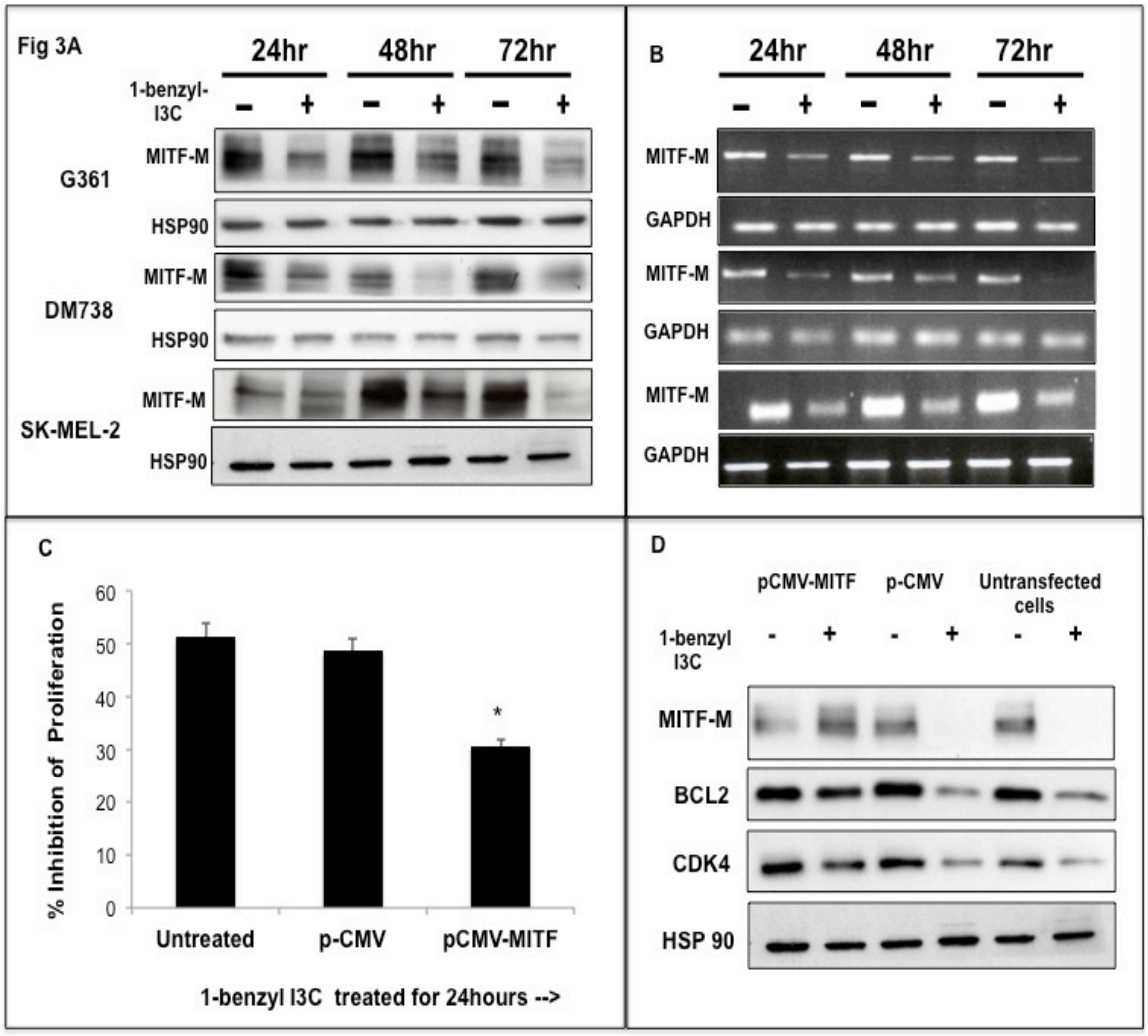


Fig 3A
24hr
48hr
72hr
1-benzyl-I3C
- + - + - +
G361
MITF-M
HSP90
DM738
MITF-M
HSP90
SK-MEL-2
MITF-M
HSP90
B
24hr
48hr
72hr
- + - + - +
MITF-M
GAPDH
MITF-M
GAPDH
MITF-M
GAPDH
C
% Inhibition of Proliferation
60
50
40
30
20
10
0
*
Untreated
p-CMV
pCMV-MITF
1-benzyl I3C treated for 24hours -->
D
pCMV-MITF
p-CMV
Untransfected cells
1-benzyl I3C
- + - + - +
MITF-M
BCL2
CDK4
HSP 90

Figure 4.

Effect of 1-benzyl I3C on Wnt signaling in cells and *in vivo* tumors.

A. BRAF-V600E expressing G-361 and DM-738 cells as well as wild type BRAF expressing SK-MEL-2 cells were treated with or without 20μM 1-benzyl I3C for 24, 48 and 72 hours. Western blots were performed on total cell extracts and probed with the indicated antibodies related to the Wnt signaling pathway.

B. 10 micron cryostat sections of tumors harvested from 1-benzyl I3C and DMSO vehicle control treated animals were analyzed for the indicated players of the Wnt signaling pathway by immunofluorescence staining. The results are 63X magnified representative images from three independent experiments.

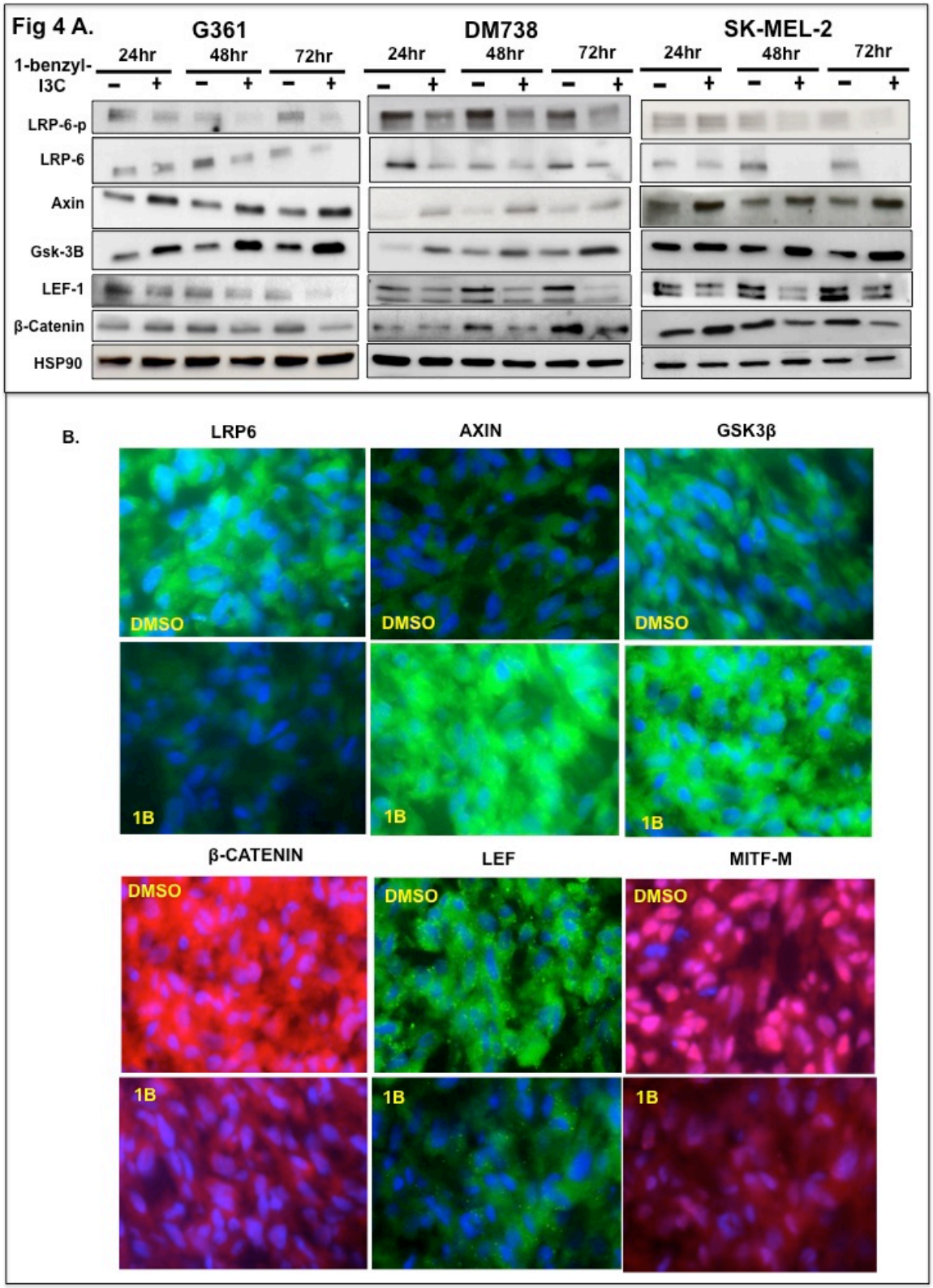


Fig 4 A.
G361
DM738
SK-MEL-2
24hr
48hr
72hr
1-benzyl-I3C
LRP-6-p
LRP-6
Axin
Gsk-3B
LEF-1
β-Catenin
HSP90
B.
LRP6
AXIN
GSK3β
DMSO
1B
β-CATENIN
LEF
MITF-M

Fig 5.

Involvement of canonical Wnt signaling in 1-benzyl I3C's mechanism of action.

A. TOP FLASH Wnt reporter assay was performed on G361 melanoma cells treated with 1-benzyl I3C for 24 hours. Cells were transfected with either an inducible TOP luciferase reporter construct driven by tandem repeats of TCF/LEF-1 response elements (TREs) or a non-inducible TRE containing FOP construct to serve as negative control. For each condition the cells were grown in Wnt conditioned media (right panel) or conditioned media with no secreted Wnt (left panel). Subsequently the cells were treated with or without 20μM 1-benzyl I3C and the luciferase activity was measured. The bar graph shows the results of three independent trials in triplicate ± SEM (*, $p < 0.01$).

B. Predictive binding simulations of 1-benzyl I3C with Wnt 5A were analyzed using the PyMol program (left side simulation). The LigPlot program examined Van der Waals interactions (within 3.5 Å) between 1-benzyl I3C and amino acids in Wnt 5A(right side simulation). (*Image courtesy: Jeanne Quirit)*

C. In order to validate the in-silico prediction, G361 cells were transfected with either a constitutively active LRP6 –luciferase reporter construct or a construct with wild type LRP6. Subsequently the cells were treated with or without 20μM 1-benzyl I3C for 24 hours and luciferase activity was determined. The bar graph shows the results of three independent trials in triplicate ± SEM (*, $p < 0.01$).

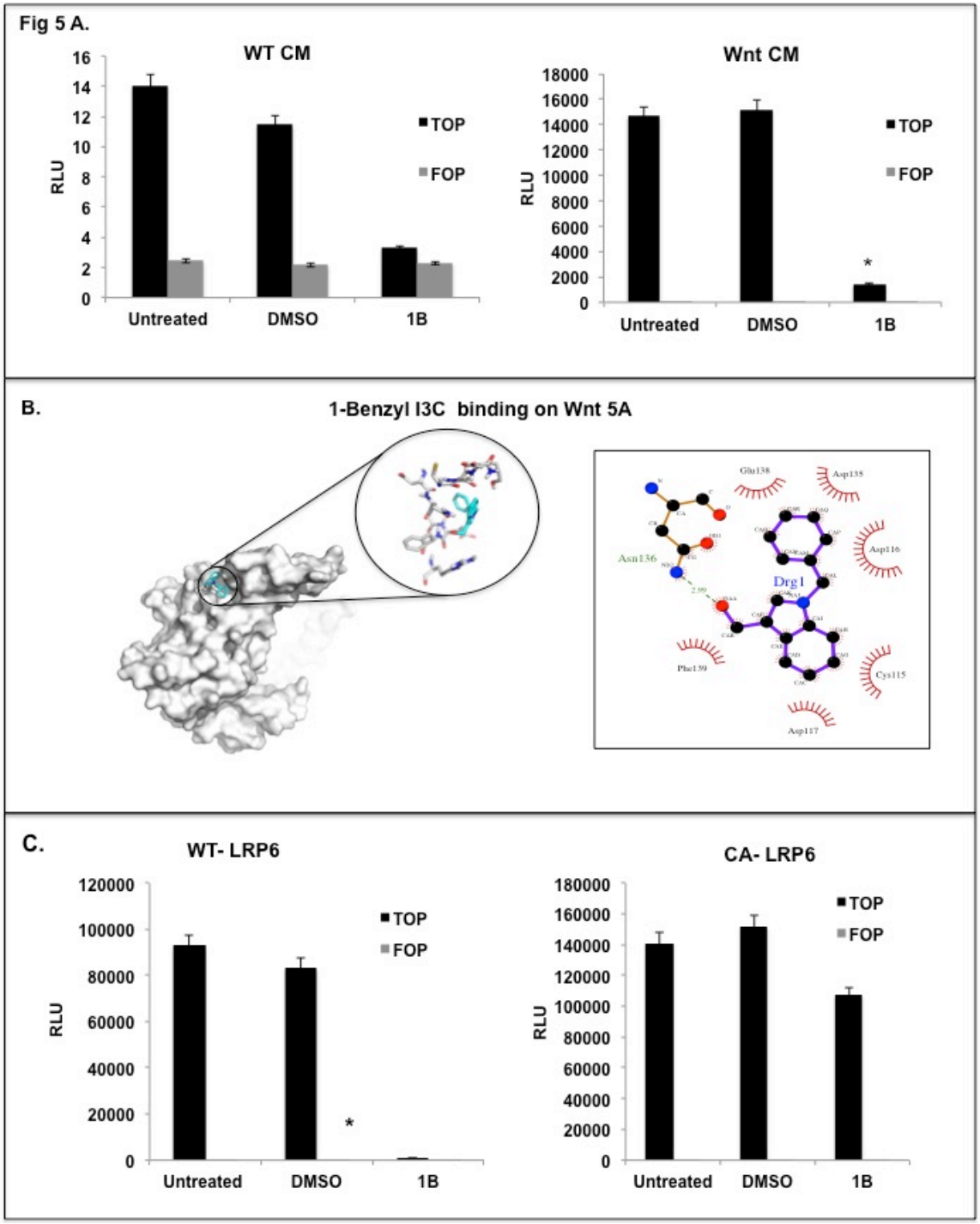


Fig 5 A.
WT CM
RLU
16
14
12
10
8
6
4
2
0
Untreated
DMSO
1B
TOP
FOP
Wnt CM
RLU
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
Untreated
DMSO
1B
TOP
FOP
B.
1-Benzyl I3C binding on Wnt 5A
Glu138
Asp135
Asn136
Drg1
Asp116
Phe139
Cys115
Asp117
C.
WT- LRP6
RLU
120000
100000
80000
60000
40000
20000
0
*
Untreated
DMSO
1B
TOP
FOP
CA- LRP6
RLU
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
Untreated
DMSO
1B
TOP
FOP

Fig 6.

Role of β-catenin/LEF1 mediated regulation of MITF-M expression in 1-benzyl I3C's mechanism of action.

A. Mutant BRAF expressing G-361 and WT BRAF expressing SK-MEL-2 cells were pre-treated with either a non-specific inhibitor - LiCl (left panel) or a specific inhibitor - BIO (right panel) of GSK3β for 3 hours. The cells were subsequently treated with or without 20 μM 1-benzyl I3C for 24 hours. Western blots were performed on whole cell extracts and probed for the indicated proteins. The results are representative of three independent experiments.

B. ChIP assay was performed on G361 cells treated with or without 20 μM 1-benzyl I3C for 48 hours using LEF-1 antibodies (IP: LEF-1) or the control IgG with one percent input as the loading control. The bar graphs quantify the densitometry results from three independent experiments ± SEM (*, $p < 0.01$).

C. Cells were transfected with reporter plasmids containing either a wild type MITF-M promoter (WT), a LEF-1 consensus site mutant (LEF-1 Mut) or PGL2 empty control vector. Luciferase specific activity was measured in cells treated with or without 20μM 1-benzyl I3C for 24 hours. The bar graph shows the results of three independent trials in triplicate ± SEM (*, $p < 0.01$).

Figure 7.

Effects of combinations of I3C and Vermurafenib on melanoma cell proliferation and BRAF-V600E signaling.

A. G-361, DM-738 and SK-MEL-2 cells were treated with the indicated combinations of 1-benzyl I3C and Vermurafenib for a suboptimal time of 24 hours and inhibition of proliferation was monitored using a CCK-8 assay. The results represent the average of three independent experiments with a mean ± SEM (*, $p < 0.01$) shown in the bar graphs.

B. G-361, DM-738 and SK-MEL-2 cells were treated with a combination of 20μM I3C and/or 10μM Vermurafenib for 24 hours and the levels of the indicated proteins determined by western blots on total cell extracts.

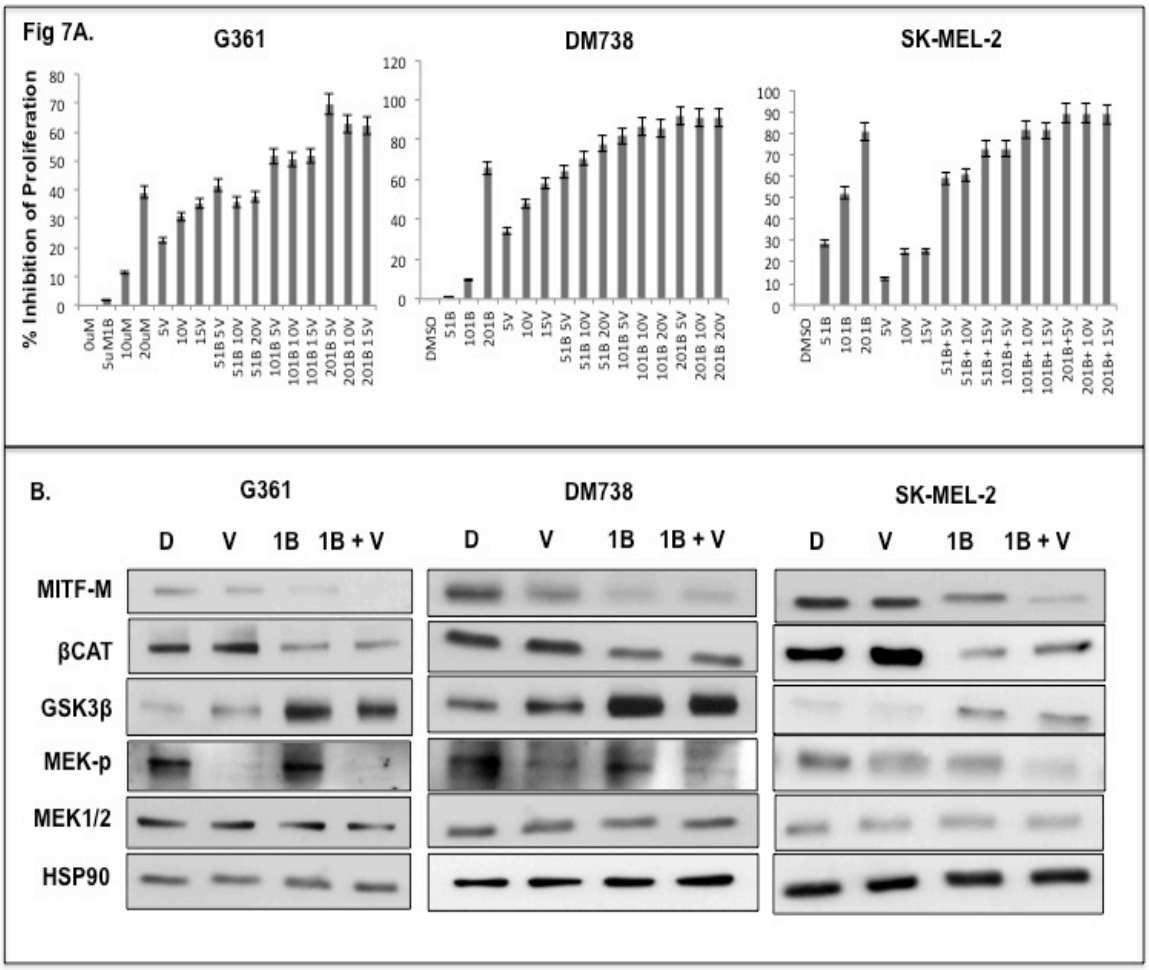


Fig 7A.
G361
DM738
SK-MEL-2
% Inhibition of Proliferation
0uM
5uM1B
10uM
20uM
5V
10V
15V
51B 5V
51B 10V
51B 20V
101B 5V
101B 10V
101B 15V
201B 5V
201B 10V
201B 15V
DMSO
51B
101B
201B
51B 5V
51B 10V
51B 20V
101B 5V
101B 10V
101B 20V
201B 5V
201B 10V
201B 20V
51B+ 5V
51B+ 10V
51B+ 15V
101B+ 5V
101B+ 10V
101B+ 15V
201B+5V
201B+ 10V
201B+ 15V
B.
G361
DM738
SK-MEL-2
D
V
1B
1B + V
MITF-M
βCAT
GSK3β
MEK-p
MEK1/2
HSP90

DISCUSSION

This preclinical study reveals 1-benzyl I3C as a novel small molecule targeting the canonical Wnt signaling pathway in human melanoma. Its high potency comparable to the clinically used melanoma drug Vemurafenib at 10 fold lower doses than the parent compound I3C, its selectivity of melanoma cells over normal epidermal melanocytes and its high tolerance in the in-vivo murine model makes 1-benzyl I3C an attractive new molecule with translational potential.

BRAF V600E/K being the most common melanoma associated mutation, the field of melanoma targeted therapy has been heavily focused on inhibiting oncogenic BRAF signaling by targeting either BRAF directly with synthetic specific inhibitors like Vemurafenib, Dabrafenib or targeting downstream components of the pathway like MEK with inhibitors like Trametinib. However prolonged treatment with BRAF pathway inhibitors led to the emergence of acquired drug resistance. Among many reported mechanisms of disease progression upon prolonged drug treatment is an overexpression of transcription factors functioning downstream of the MAP Kinase pathway like MITF-M which confers an oncogenic melanocytic lineage dependency causing resistance [16]. In another study MITF-M expression was found to be sufficient to render melanoma cells resistant to MEK or ERK inhibitor [17,18].

Besides MITF-M, the Wnt signaling pathway too has been recently implicated in therapeutic escape [23]. MEK inhibitor resistance has been reported to display a signature associated with increased Wnt signaling evident from upregulated β-catenin expression [24]. Additionally inhibition of Wnt/ β-catenin pathway in melanoma cell lines has shown attenuation of proliferation [25]. Wnt5A has been extensively implicated in melanomagenesis and progression controlling cell polarity, orientation, and directional movement in melanoma cells [26]. Elevated Wnt5a expression promotes cell motility and drives metastasis and direct inhibition of Wnt5A has been shown to decrease adhesion, invasion, and migration in melanoma cell line [27]. The Wnt/β-catenin signaling pathway is aberrantly activated in one third of melanomas with the subset showing very poor prognosis. Yet this pathway does not show any disease causing primary mutations [28].

In this scenario 1-benzyl I3C is a first of its kind indolebased small molecule that directly inhibits Wnt signaling in melanoma cells to downregulate expression of MITF-M resulting in anti-proliferation. This possibly accounts for 1-benzyl I3C's effectiveness over a much wider range of melanoma cell lines than I3C or Vemurafenib whose effectiveness is largely restricted to the presence of oncogenic BRAF. We have previously shown that I3C also disrupts MITF-M expression in melanoma cells but in an oncogenic BRAF-dependent way through BRN-2. Interestingly addition of the benzyl group at position 1 alters the direct binding target of the molecule and the signaling pathway it hits. The mechanisms of action of both compounds however converge at a common node with MITF-M to result in an overall antiproliferative effect in melanoma. This rehashes the emerging concept from our studies that these natural and synthetic indolecarbinol compounds bring about their anti-cancer effects through different direct binding targets that are usually upregulated oncogenic proteins and hence their mechanism of

action is specific yet context dependent. This also imparts functional versatility to these molecules and widens their clinical potential significantly.

Currently the therapeutic options for wild type BRAF melanoma patients are limited by the heterogeneity of the disease and the lack of identified driver mutations. About one third wild-type BRAF patients have NRAS mutations, a very small subset of patients express cKit mutations and some others show BRAF, non-V600 mutations or BRAF fusion kinases [29]. Although NRAS is a known cancer causing driver mutation, it has been a challenge to get a drug that hits the target and effectively blocks it. MEK inhibition alone has also been reported to be insufficient to treat wild-type melanomas. A study in a mouse model using MEK inhibitor melanomas produced apoptosis but did not trigger a cell cycle arrest [30]. At present effective single agent targeted therapy is available for wild type melanomas and it is likely that other agents combined with MEK inhibitors will be a direction of treatment. 1-benzyl I3C in this respect represents a unique small molecule that causes a G1 cell cycle arrest and antiproliferation in SK-MEL-2 cells expressing wild-type BRAF and holds out promise for clinical use to treat wild-type BRAF melanomas.

Additionally combination of 1-benzyl I3C and Vemurafenib display a co-operative antiproliferative effect greater that each compound alone. A therapeutic strategy that is gaining momentum in the field of current melanoma therapy is the use of combinations of drugs with minimal side effects, to target either multiple players of the same signaling cascade. For example treatment with combinations of the BRAF inhibitor Dabrafenib (Trafilnar) and the MEK inhibitor Trametinib (Mekinist) has shown improved overall survival in patients leading to FDA approval of the combination for advanced melanoma [31,32]. Trials with combinations of small molecules and immunotherapy have also shown positive outcome. This trend of combination therapy has also extended from small molecule targeted therapy to immunotherapy for the treatment of melanoma. Recently a Phase III trial has shown that the combination of nivolumab- a monoclonal antibody against PD-1 and ipilimumab – a monoclonal antibody against CTLA-4, significantly increased progression-free survival (PFS) in patients with advanced melanoma compared with ipilimumab alone [33].

Therefore 1-benzyl I3C can be used as a single agent therapeutic molecule or potentially combined with other BRAF pathway inhibitors to bring about a more robust antiproliferative effect by targeting multiple aberrant pathways. This combination could also delay or prevent the emergence of drug-induced resistance in melanoma thereby optimizing the efficacy of targeted therapy.

The aberrant activation of the Wnt signaling pathway in a vast range human malignancies underscore its relevance and also the importance of being able to target it effectively for therapeutic outcomes. Nearly 90% of colon cancers harbor mutations in the Wnt signaling pathway either inactivating APC or activating β-catenin [34-36]. Interestingly recent evidence has also illustrated a critical role of β-catenin in cancer stem cells (CSC)s [37-39]. For example, stem-like colon cells with elevated β-catenin signaling have significantly greater tumorigenic potential than cells with low β-catenin signaling. Further Wnt signaling has been reported in Hematopoieic stem cells (HSC) and in their niche microenvironments regulating their function

[40-44]. It is therefore reasonable to hypothesize that the potential of 1-benzyl I3C as an anti-cancer molecule is not restricted to melanoma alone.

FUTURE DIRECTIONS

Evaluation of melanoma biopsy samples for Indole sensitivity

To take the findings of this dissertation to the next level of clinical translation, melanoma biopsy samples from melanoma patients with different grades of disease, different mutation profiles and different treatment history should be obtained and tested for their sensitivity to I3C, 1-benzyl I3C as well as their combinations with clinically used drugs like Vemurafenib, Dabrafenib etc. Primary cell cultures can be established from biopsy samples and tested for their indole-responsiveness. Sensitive cell-lines can be further injected in athymic nude mice to generate xenografted tumors and tested for indole-sensitivity. This approach will aid in verifying if the mutation profile dependent effect of I3C, effectiveness of 1-benzyl I3C at clinically relevant doses and their combinations with other drugs on melanoma cells as observed in cell culture and in melanoma xenografted murine model in this body of work can be translated in actual patients. Additionally this could provide information about other correlates of Indole-sensitivity like stage or grade of melanoma or treatment history, which was beyond the scope of the current study.

***In-vivo* experiments**

The effectiveness of I3C and 1-benzyl I3C as monotherapeutic agents against human melanoma has been demonstrated in the context of a whole organism using the xenografted murine model in this dissertation. However the combinatorial effect of these two compounds with Vemurafenib has been tested only in cell cultures. Therefore a logical preclinical extension of this study would be to test if the cooperative antiproliferative effect of I3C or 1-benzyl I3C with Vemurafenib would effectively translate in a whole animal. This can be done by generating melanoma cell xenografted tumors in immunocompromised athymic nude mice as described in this dissertation and subsequently treating the tumors with an optimum combination of I3C and Vemurafenib or 1-benzyl I3C and Vemurafenib over an extended period of time. Additionally new combinations of I3C and 1-benzyl I3C can also be efficiently tested using this murine model to discover better, more potent indole combinatorial therapy for melanoma.

Different sites of administration of these compounds either as single agents or as combinations can also be tested using this murine model to determine the most effective method of delivery. The study described in this dissertation involved subcutaneous scruff injection for delivery of the compounds under consideration. However since unlike most cancers melanomas are superficial and easily accessible, direct delivery of the compound by injections at the site of malignancy is a method that should be tested for better treatment outcome.

Finally, this in-vivo xenografted murine model of melanoma can also be used to test the effectiveness of I3C or 1-benzyl I3C and their combinations with other drugs against development of acquired drug resistance upon prolonged treatments. An alternative murine model of drug resistance that can be used is an inducible melanoma model - Tyr::CreERT2;PTEN$^{F/F}$;BRAF$^{F-V600E/+}$ that has been specifically developed to preclinically identify efficient treatment combinations. Tumors from this model harbor the BRAFV600E mutation and are PTEN-deficient, making them highly suitable for the testing of targeted therapies. One of the reported mechanisms of development of drug resistance to prolonged BRAF specific inhibitor treatment is compensatory signaling through other oncogenic pathways

like PTEN/AKT-1, Wnt/β-catenin. Since I3C targets both oncogenic BRAF and stabilizes PTEN and 1benzyl I3C inhibits the Wnt pathway, there is an intriguing possibility that these compounds by themselves or in combination with other drugs might delay or even abrogate development of resistance upon prolonged treatment.

Testing direct binding of 1-benzyl I3C to Wnt

In-silico computer aided molecular binding simulations using the known 3-D crystal structures of components of the Wnt signaling pathway like LRP6, Frizzled and Wnt showed a putative direct binding pocket of 1-benzyl I3C on the Wnt ligand itself. Predictive binding of 1-benzyl I3C to both LRP6 and Frizzled are superficial and the interactions not unstable. This preliminary result can be substantiated by in –vitro direct binding assays using purified human Wnt, like Isothermal Titration Calorimetry to test if 1-benzyl I3C directly binds to the Wnt ligand.

Extending to other cancers and stem cells

The discovery of using Indole-3-carbinol and its synthetic derivatives as single agents as well as in combination therapy with other drugs in melanoma, can be extended to treat other type of skin cancers as well, like squamous and basal cell carcinoma and keratocanthomas, which also express oncogenic BRAF and shows upregulation of Wnt signaling.

The scope of this discovery can be further expanded to test the effect of I3C on other types of human cancers expressing BRAF mutations, such as renal cancer or cancers that show aberrations in the Wnt signaling pathway like hepatocellular carcinoma, colorectal, kidney cancers, leukemia, lymphoma and breast cancers or cancers expressing inactivating mutations in PTEN like endometrial, prostrate and central nervous system cancers.

Recent reports have implicated Wnt signaling in a critical role not only in cancer but also in cancer stem cells/tumor initiating cell populations with aberrant Wnt signaling being associated with a wide array of tumor types. Interestingly, many cell surface markers like LGR5/GPR49, CD44, CD24, and Epcam that have been used to identify and isolate putative tumor stem cell populations in a variety of tissues are direct targets of Wnt as well. The role of the Wnt signaling cascade, particularly in other malignancies, which do not carry classical activating mutations in the Wnt pathway like multiple myeloma have also become apparent from recent research. However, there are only a limited number of Wnt therapeutics that are either in early development or in Phase 1 clinical trial to target cancer or cancer stem cells. Hence studies can be designed to test the effect of 1-benzyl I3C on cancer stem cells as well as other Wnt related disord

CONCLUSION

Our studies show I3C and its highly potent derivative – 1 Benzyl I3C as a novel class of potential therapeutic compounds for treatment of human melanomas. The synthetic specific inhibitors currently available in the market for melanoma-targeted therapy inhibits single oncogenic pathways and upon prolonged treatment results in drug induced acquired resistance, where-in another alternative oncogenic pathway is hyperactivated to circumvent the drug-induced inhibition. I3C on the other hand is a single agent that simultaneously targets multiple oncogenic pathways, which can possibly delay or prevent development of drug resistance. Our experiments combining I3C with Vemurafenib also show that, since I3C has a different and hence non-competing binding site from most clinically used BRAF V600E inhibitors and has been clinically shown to be very well tolerated, I3C could be effectively used in combination with currently used drugs to treat melanoma.

Importantly, the easy, over the counter availability and the low cost of I3C gives our discovery a significant competitive edge over other prohibitively expensive clinically used drugs. The current cost of research grade I3C sold by Sigma Aldrich is $44/gram and the cost of I3C food supplement available in the market on an average is $1.2 – 2.00/gram. In stark contrast the average price of currently used melanoma drugs like Vemurafenib/Zelboraf is $200/gram and the with current treatment regimes the cost/month for most of the commonly prescribed drugs range between $9000-$13,000/month [54]. Given the substantially lower cost of I3C compared to most currently available melanoma therapeutics, this treatment option may be lucrative for potentially interested commercial parties like Biopharmaceutical companies. For patients it might be an economical option as primary single agent or combination therapy as well as an adjuvant therapy depending on the stage and grade of the disease.

1-benzyl I3C targets the Wnt signaling pathway, which has recently been implicated in melanoma progression, tumor survival, metastasis as well as chemoresistance. Currently there are only a few Wnt pathway inhibitors in Phase I clinical trial but none being tested on melanoma. More importantly none have been currently approved for clinical use. Therefore our findings make 1-benzyl Indole-3-carbinol a novel, first in class, small molecule indolecarbinol derivative that inhibits Wnt signaling in the context of human melanoma. Preclinical evidence from our lab shows 1-benzyl Indole-3-carbinol is well tolerated in a xenografted murine model of human melanoma and successfully attenuates tumor growth, at a dose more than 10 fold lower than I3C and comparable to concentrations of clinically used drugs for melanoma therapy. Additionally unlike I3C, since 1-benzyl I3C's action is not restricted by the presence of an oncogenic protein like BRAF V600E, it showed effectiveness over a broader range of melanoma phenotypes. However 1-benzyl I3C has never been tested for clinical use to date. We therefore propose 1-benzyl I3C as a promising candidate for clinical trials for treatment of malignant melanoma.

Unlike most cancers, which are internal and therefore can be accessed only by invasive methods, melanoma has easier site accessibility being largely cutaneous in nature. Keeping this in mind we also propose a novel dermal or transdermal delivery method for I3C as well as 1-benzyl I3C using either a topical preparation or dermal/ transdermal delivery patches. Neither I3C, 1-benzyl I3C has been previously administered by any such direct routes of delivery for the treatment of malignant melanoma or any other skin condition. The advantage of this approach is ease of administration and, if required, termination of treatment, as well as direct contact of the drug to

the site of malignancy. Importantly for I3C, this direct on site administration would aid to circumvent the issue of dimerization of I3C into DIM, as a result of acid condensation reaction in the stomach in an oral route of delivery method. Unlike common thought in the field, preliminary evidence in our lab shows DIM and I3C has different molecular mechanism of action and to harness I3C's inhibitory effect on BRAFV600E's enzymatic activity it would be advantageous to get direct access to the site of disease. As for 1-benzyl I3C, even though its has been shown to be stable in acidic environments like the stomach because of the added 1-benzyl moiety to the imidazole ring nitrogen prevents self condensation, however, the metabolic transformation of 1-benzyl-I3C has not been thoroughly investigated. Hence using dermal/ transdermal delivery can only minimize off-target systemic effects of potential indolecarbinol metabolic intermediates.

Overall, our discovery demonstrates the potential for development of Indolecarbinol based natural bioactive compounds like Indole-3-Carbinol and its synthetic derivative like 1-benzyl Indole-3-carbinol as a novel class of efficacious and economical small molecule for targeted therapy of melanoma as well as other dermatological conditions and cancers. This can present commercial interest for Biopharmaceutical companies to invest in for further development for clinical use and thereby offer a novel therapeutic approach for physicians and patients to treat a variety of cancers and disease condition.

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