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Cooperative anti-proliferative signaling by aspirin and indole-3-carbinol targets micropthalmia associated transcription factor gene expression and promoter activity in human melanoma cells

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Abstract

Anti-proliferative signaling of combinations of the nonsteroidal anti-inflammatory drug acetylsalicylic acid (aspirin) and indole-3-carbinol (I3C), a natural indolecarbinol compound derived from cruciferous vegetables, was investigated in human melanoma cells. Melanoma cell lines with distinct mutational profiles were sensitive to different extents to the anti-proliferative response of aspirin, with oncogenic BRAF-expressing G361 cells and wild type BRAF-expressing SK-MEL-30 cells being the most responsive. I3C triggered a strong proliferative arrest of G361 melanoma cells, and caused only a modest decrease in proliferation of SK-MEL-30 cells. In both cell lines, combinations of aspirin and I3C cooperatively arrested cell proliferation and induced a G1 cell cycle arrest, and nearly ablated protein and transcript levels of the melanocyte master regulator Micropthalmia Associated Transcription Factor isoform M (MITF-M). In melanoma cells transfected with a −333/+120 bp MITF-M promoter-luciferase reporter plasmid, treatment with aspirin and I3C cooperatively disrupted MITF-M promoter activity, which accounted for the loss of MITF-M gene products. Mutational analysis revealed that the aspirin required the LEF1 binding site, whereas, I3C required the BRN2 binding site to mediate their combined and individual effects on MITF-M promoter activity. Consistent with LEF1 being a down-steam effector of Wnt signaling, aspirin, but not I3C, down-regulated protein levels of the Wnt co-receptor LDL receptor-related protein-6 and β-catenin and up-regulated the β-catenin destruction complex component Axin. Taken together, our results demonstrate that aspirin-regulated Wnt signaling and I3C-targeted signaling pathways converge at distinct DNA elements in the MITF-M promoter to cooperatively disrupt MITF-M expression and melanoma cell proliferation.

Keywords

Aspirin; Indole-3-carbinol; LEF1; Melanoma cells; Micropthalmia associated transcription factor; Wnt signaling

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Introduction

Human melanomas, which arise from melanocytes of neuro-ectodermal origin, are the most aggressive form of malignant skin cancers, and can be categorized by distinct mutational profiles that determine their corresponding cellular phenotypes, proliferative capabilities, and therapeutic options (Hawryluk and Tsao et al, 2014). In approximately 60% of melanomas, mutations within the *BRAF* gene result in constitutively activation of the Ser/Thr protein kinase with oncogenic properties (Davies et al, 2002). Signaling by oncogenic BRAF leads to hyper activation of MEK and Erk/MAPK, and in recent years new therapeutic approaches have been developed that target specific components of this cellular cascade, such as the oncogenic BRAF inhibitors Vemurafenib (Sosman et al, 2012) and Dabrafenib (King et al, 2013) as well as the MEK inhibitor Trametinib (Kim et al, 2013). A critical down stream effect of the oncogenic BRAF signaling is enhanced expression levels of Microphthalmia Associated Transcription Factor isoform M (MITF-M), a basic helix-loop-helix leucine zipper transcription factor highly involved with cell processes of the melanocyte lineage (Regad, 2013). Through its transcriptionally regulated gene products, MITF-M plays an essential role in cell survival, morphology, migration, differentiation and proliferation of melanocytes and has a complex role in the malignant transformation, progression, proliferation and metastasis of melanoma (Levy et al, 2006, Pierrat et al, 2012). Enhanced levels of MITF-M is considered to be a critical factor in determining the efficacy and treatment outcomes of a given melanoma therapy and probability of relapse of the disease (Garraway et al, 2005). Therefore, an essential foundation to develop new pre-clinical therapeutic strategies for melanoma is to assess effects on MITF-M; however, a mechanistic understanding of the regulation of MITF-M gene expression by potential anti-cancer agents is not well understood.

Dietary phytochemicals and their synthetic analogues represent an intriguing group of compounds with the potential to regulate melanoma tumor growth and spread of malignancies through multiple cellular pathways with minimal side effects (Strickland et al, 2015, Surh, 2003, Sarker et al., 2009). One such molecule is Indole-3-Carbinol (I3C), a naturally occurring compound derived from glucobrassicin made in cruciferous vegetables of the *Brassica* genus, such as broccoli, cauliflower, and Brussels sprouts (Aggarwal and Ichikawa, 2005, Ahmad et al, 2012). I3C has been shown to trigger anti-proliferative and pro-apoptotic properties *in vivo* and in a variety of cultured human cancer cells by controlling specific transcriptional, enzymatic, metabolic, and growth factor and hormone-dependent cell signaling processes (Firestone and Bjeldanes et al, 2003, Firestone and Sundar et al, 2009, Marconett et al, 2010, Marconett et al, 2011, Maruthanila et al, 2014, Sarkar et al, 2009, Xu et al, 2011). In clinical trials, I3C was tolerated well with minimal side effects (Reed et al, 2005). From a mechanistic viewpoint, we have established that the presence of specific I3C target proteins expressed in human cancer cells, such as Human Neutrophil Elastase (Nguyen et al, 2008, Aronchik et al, 2010, Aronchik et al, 2012) in breast cancer and the Neural precursor cell Expressed Developmentally Down-regulated 4 (NEDD4-1) a ubiquitin ligase whose function is critical in a subset of melanoma genotypes (Aronchik et al, 2014). We recently observed that I3C strongly inhibits oncogenic BRAF enzymatic activity with little effect on the wild type enzyme under conditions in this

indolecarbinol disrupts proliferation of oncogenic BRAF-expressing melanoma cells (Kundu et al., 2016). Inhibition of these target proteins' activities, and possibly others, mediates the efficacy by which I3C selectively stimulates distinct anti-proliferative signaling cascades (Aronchik et al, 2010, Aronchik et al, 2012). Other studies have shown that I3C treatment increased sensitivity to UV induced apoptosis and enhance cytotoxic responses in human melanoma (Kim et al, 2006, Kim et al, 2011) and squamous cell (Cope et al, 2006), respectively. Also, ectopic application of I3C directly inhibits skin tumor formation in mouse models (Srivastava and Shukla, 1998). We have observed that human melanoma cells with distinct mutational profiles are sensitive to the anti-proliferative effects of I3C (Aronchik et al, 2014), suggesting that this natural phytochemical could provide one component of a combinational therapy for human melanomas.

Another molecule that was originally discovered from plants that has anti-cancer properties and is safely ingested by humans is acetylsalicylic acid (aspirin). Daily intake of aspirin has been shown to significantly reduce overall tumor burden in cancer patients (Rothwell et al, 2012). For example, long-term ingestion of low doses of aspirin reduced the incidence of melanoma in women (Gamba et al, 2013), and other clinical studies showed lower rates of colorectal, esophageal, pancreatic, and lung cancer in patients taking aspirin (Rothwell et al, 2011, Zhang et al, 2015). The cellular mechanism of aspirin action accounting for its anti-cancer effects has been investigated in several cancer cell model systems including melanoma cells (Cheng et al, 2012, Vad et al, 2008). Recent studies have pointed to aspirin's ability to inhibit Cyclooxygenase 1 and 2 (COX-1/2) enzymatic activity as playing a role in the anti-proliferative effects of aspirin (Chan et al, 2007, Xin et al, 2007). In addition, aspirin treatment leads to the disruption of Nuclear Factor Kappa-light-chain enhancer of activated B cells signaling, and activation of apoptotic pathways (Kopp and Ghosh, 1994, Sharma et al, 2010, Yin et al, 1998). Despite these observations, aspirin's mechanisms of action outside of COX-1/2 inhibition have not been studied in depth in melanoma and nothing is known about the potential effects of aspirin on MITF-M levels.

Multiple cell signaling pathways converge on the MITF-M promoter, suggesting that this critical regulator of melanoma proliferation can be used to assess potential effectiveness at a pre-clinical level of newly developed therapeutic strategies for melanoma. In this study, we now demonstrate that a combination of I3C and aspirin cooperatively arrest proliferation and induce a G1 cell cycle arrest of human melanoma cells and nearly ablate MITF-M gene expression. Aspirin and I3C anti-proliferative signaling converges on the MITF-M promoter through the Lymphoid Enhancer-binding Factor 1 (LEF1) binding site, a down stream effector of the Wnt pathway (Takeda et al, 2000), and the Brain-2 transcription factor (BRN2) binding site, a down stream effector of the BRAF-MEK-MAPK signaling cascade (Wellbrock et al, 2008), respectively. Our study expands on the limited mechanistic understanding on aspirin's mode of action in melanoma cells as well as implicating at a pre-clinical level a combination of aspirin and I3C as a new potential therapeutic strategy for melanoma.

Materials and Methods

Reagents and cell culture

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

Cell proliferation assay

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

Flow cytometry

Cells were plated onto six-well tissue culture plates at 70% confluency and treated as indicated in triplicate with the medium changed every 24 hours. Following the treatment the cells were washed once with phosphate buffered saline (PBS) (Lonza) and harvested with 1mL of PBS.

Cells were then lysed in 300μL of DNA staining solution (0.5mg/mL propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100). Nuclear fluorescence of wavelength more than 585nm was measured on a Beckman-Coulter EPICS XL instrument with laser output adjusted to deliver 15 megawatts at 488nm. For each sample 10,000 nuclei were analyzed and the percentage of cells in G_1, S, G_2/M phase of the cell cycle was determined by analysis with Multicycle provided by Phoenix Flow Systems in the Cancer Research Laboratory Flow Cytometry Facility of the University of California, Berkeley.

Western blots

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

Western blotting and antibody diluations were carried out as previously described (Sundar et al, 2006), and proteins were visualized using Enhanced Chemiluminescence Lightning reagent (GE Healthcare, Piscataway, NJ) on nitrocellulose membranes. Mouse anti-MITF antibodies (Thermo-Fisher, #MA5-14146) was diluted 1:200 in wash buffer and the Rabbit anti-CDK2 (Santa Cruz Biotechnology, Inc., Santa Cruz CA, #sc-163) was diluted 1:500 in wash buffer.

Reverse transcription-polymerase chain reaction

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

Transfections and luciferase reporter plasmid assays

The pGL2-333/+120-MITF-M promoter-luciferase reporter plasmid was a kind gift from Dr. Richard Marais (Cancer Research UK Centre of Cell and Molecular Biology, London, United Kingdom) (Wellbrock et al, 2008). G361 and SK-MEL30 cells were grown to 70% confluence in six-well plates (Nunc) and transfected with 2 μg/well DNA of the indicated plasmid construct. Transfections were performed using either Superfect (Qiagen) for G361 cells or Fugene6 (Promega, Madison, WI) for SK-MEL-30 cells as per manufacturer's instructions. A ratio of 2 Superfect:1μg DNA was used for transfecting G361 cells and 4 Fugene6:1μg DNA for SK-MEL-30 cells. Cells were treated 24 hours post-transfection as indicated for 48 hours. Cells were then harvested and lysed and relative luciferase activity was evaluated using the Luciferase assay kit (Promega). Relative luciferase activities were normalized to protein input as determined by the Bradford protein assay. These results were verified by three independent experiments performed with triplicate samples of each treatment.

Generation of MITF-M promoter mutations

Mutations were generating utilizing the pGL2-333/+120-MITF-M as a template. pGL2-333/+120-MITF-M-ΔLEF1(ΔLEF1) was generated using the following primers: LEF1 forward, 5′-GACAGTGAGTTTGACTTTGGCAGCTCGTCACTTAA-3′ LEF1 reverse 5′-TTAAGTGACGAGCTGCCAAAGTCAAACTCACTGTC-3′. pGL2-333/+120-MITF-M-ΔBRN2(ΔBRN2) was generated using the following primers: BRN2 forward, 5′-TACATGCATAACTAGCGAGCTTAGGTTATTATAAGC-3′ BRN2 reverse, 5′-GCTTATATTAACCTAAGCTCGCTAGTTATGCATGTA-3′. The following PCR conditions

were utilized;-ΔLEF1-2 min at 95°, 30 s at 95°, 30 s at 59°, 8 min at 72°, for 30 cycles, −ΔBRN2-30 s hotstart at 95°, 30 s at 95°, 1 min at 55°, 8 min at 68°, for 16 cycles. −ΔLEF1-ΔBRN2 Double mutant was made by utilizing the −ΔBRN2 mutant as a template and inserting the LEF1 mutation as described above. Mutagenesis was performed using QuickChange II kit (Aligent) per the manufacturer's instructions. PCR products were extracted and purified using QIAquick Gel Extraction Kit (Qiagen). Sequence was confirmed by automated DNA sequencing (University of California Berkeley Sequencing Facility).

Measurement of PGE_2 levels by enzyme-linked immunosorbent assay

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

Melanoma cell Invasion Assay

Cell invasion was determined using BioCoat Matrigel Invasion Chambers (Corning Inc., New York) according to manufacturer instructions. Briefly, 5×10^4 cells/ml were added in triplicate to the upper well in with serum free medium containing the DMSO vehicle control, 50μM I3C, 1.5mM aspirin, or 50μM I3C and 1.5mM aspirin. Full medium containing 10% FBS as a chemoattractant was added to the bottom wells. After incubation at 37°C 5% CO_2 for 22 hours, non-invading cells were removed from upper surface by scrubbing, and cells that invaded through the Matrigel membrane were fixed and stained with 100% methanol and 1% Toluidine blue, respectively. Cells were then counted under a light microscope.

Results

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

Human melanoma can be categorized by their mutational profiles, including expression of oncogenic forms of BRAF and NRAS, and mutations in tumor suppressor proteins such as PTEN and p53, which determine the corresponding cellular phenotypes, proliferative capabilities and therapeutic options (Gray-Schopfer et al, 2007). Therefore, to initially examine the potential anti-proliferative effects of aspirin, five different human melanoma cell lines (G361, SK-MEL-30, DM-738, SK-MEL-2, and SK-MEL-28 cells) were treated with increasing concentrations of aspirin, up to 6 mM, for 48 hours and the cell viability determined using a CCK-8 assay. Cells treated with only the vehicle control represented the 100% cell viability level for each cell line. As shown in Figure 1, in all cell lines tested, aspirin caused a dose dependent decrease in total cell number compared to the vehicle control, although the efficacy of this anti-proliferative response differed somewhat between each of the tested melanoma cell lines. G361 and SK-MEL-30 cells were the most sensitive to aspirin treatment, with the half-maximal response occurring at approximately 3.0 mM aspirin (Figure 1), whereas, the other tested melanoma cell lines required higher concentrations of aspirin to induce a less pronounced anti-proliferative effect. There was no

strong correlation between the overall mutagenic profiles and sensitively to aspirin, although the most sensitive melanoma cell lines express wild type forms of PTEN and p53. In a generally similar experiment, the anti-proliferative effects of 48-hour treatment with 200 μM I3C was assessed in each of the melanoma cell lines. G361 melanoma cells were the most sensitive cell line to the anti-proliferative effects of I3C, whereas, the other four cell lines were partially sensitive to the anti-proliferative effects of I3C (Figure 1, lower right panel). Therefore, because of their differential responsiveness to the anti-proliferative effects of aspirin and I3C and their distinct mutagenic profiles, G361 and SK-MEL-30 cells were used and compared throughout our study.

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

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

One potential down stream target of aspirin and I3C anti-proliferative signaling is MITF-M because this transcriptional regulator activates several critical melanoma cell pathways by regulating expression of genes directly involved in cell cycle progression, cell migration, and inhibition of apoptosis (Yajima et al, 2011). Therefore, the effects of 48-hour treatments with combinations of aspirin and/or I3C on MITF-M protein levels were examined in both G361 and SK-MEL-30 human melanoma cells by western blot analysis. As shown in Figure 2b, in both melanoma cell lines, aspirin treatment had only a modest effect on MITF-M protein levels compared to vehicle control treated cells. In contrast, I3C strongly down-regulated MITF-M protein levels in G361 cells but had only a minimal effect on MITF-M levels in the SK-MEL-30 cells. Similar to effects on cell proliferation, treatment with sub-maximal combinations of both aspirin and I3C strongly down-regulated MITF-M protein levels in both melanoma cells well beyond the effects observed in cells treated with either compound alone (Figure 2b). This synergistic down regulation of MITF-M protein levels was first observed in G361 cells treated a combination of 1.0 mM aspirin and 50 μM I3C. Densitometric analysis demonstrated that I3C and aspirin treatment alone only reduced MITF-M levels by 7% and 2% respectively, whereas, treatment with the combination

reduced MITF-M levels by 28%. This cooperative effect was even more pronounced upon treatment with 50 μM I3C and 1.5 mM aspirin, in which the individual treatments reduced protein levels by 7% and 20% respectively, but the combination nearly ablated the production of MITF-M protein. Similar effects were observed with the SK-MEL-30 cells under the same treatment conditions with the first observable synergistic effect on MITF-M protein levels was found upon treatment with a combination of 50 μM I3C and 2.0 mM aspirin. Treatment with a combination of 3.0 mM aspirin and 150 μM I3C strongly disrupt production of MITF-M protein. Taken together, these results demonstrate that compared to the effects of each compound alone, a combination of aspirin and I3C displays a significantly more potent anti-proliferative response including the loss of MITF-M protein production.

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

MITF-M binds to the promoters and stimulates transcription of several cell cycle genes to promote melanoma cell proliferation (Carreira et al, 2005). One such critical cell cycle gene that is a target of MITF-M transcriptional activity is Cyclin Dependent Kinase 2 (CDK2), which contains an MITF-M DNA binding site (CATGTG) in its promoter at −1315 base pairs upstream of the transcriptional start site (Du et al, 2004) (see Figure 3a diagram). To functionally test the down stream effects of aspirin and I3C down regulation of MITF-M protein levels, the effects of 48-hour treatments of G361 cells with combinations of aspirin and/or I3C on CDK2 transcript levels was examined by RT-PCR analysis. As shown in Figure 3a, CDK2 transcript levels were modestly attenuated by treatment with I3C or aspirin in a dose dependent manner. When the cells were treated with combinations of the two compounds, a synergistic decrease of CDK2 mRNA levels was observed that generally correlated in a dose-dependent manner to the cooperative effect on MITF-M protein levels. Expression of CDK2 transcripts was almost completely abolished upon treatment with 3.0 mM ASA and either 50 μM or 150 μM I3C (Figure 3a), which are concentration combinations that ablated MITF-M protein production in G361 cells (Figure 2b, lower right gels of left panel).

The loss of MITF-M protein levels and down-regulation of its CDK2 target gene predicts that combinations of aspirin and I3C should strongly induce a cell cycle arrest of G361 and SK-MEL-30 melanoma cells. To test this possibility, both cell lines were initially treated for 48 hours with indicated concentrations of aspirin and I3C alone, and DNA content determined by flow cytometry analysis of propidium iodide stained nuclei. As shown in Figure 3b, consistent with a G1 cell cycle arrest, treatment of both melanoma cell lines with either aspirin or I3C dose-dependently induced an increase in G1 phase DNA content with maximum effects observed at 3.0 mM aspirin and 200 μM I3C, respectively. Similar to effects on MITF-M levels, G361 melanoma cells displayed a stronger G1 cell cycle arrest compared to the SK-MEL-30 melanoma cells. To assess the potential synergistic effects of aspirin and I3C, cells were treated for 48 hours with different concentration combinations of these compounds and DNA content analyzed by flow cytometry analysis of propidium iodide stained nuclei. At all of the combinations of sub-maximal concentrations of each compound, treatment with aspirin and I3C synergistically induced a G1 cell cycle arrest,

compared to cells treated with the compounds alone (Figure 3c, left panel). The combination of 3.0 mM aspirin and 150 μM I3C, both maximal concentrations, strongly induced a G1 cell cycle arrest that was only slightly greater than the effects of each compound alone. A generally similar effect was observed with the SK-MEL-30 melanoma cells, although the final percentage of the cell population arrested with a G1 DNA content was less than observed with the G361 cells. Taken together, these observations indicate that combinations of aspirin and I3C effectively down regulate MITF-M protein levels, causing the loss of CDK2 expression, resulting in a G1 cell cycle arrest of the melanoma cells.

I3C and aspirin down regulate MITF-M transcript levels and promoter activity

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

To determine if the aspirin and I3C down regulation of MITF-M transcript levels can be accounted for by decreased MITF-M promoter activity, both melanoma cell lines were transiently transfected with a MITF-M promoter-reporter plasmid containing the -333 bp upstream of the RNA start site (pGL2-333/+120-MITF-M). This construct was previously demonstrated to be active in transfected melanoma cells (Wellbrock et al, 2008), and contains multiple transcription factor binding sites, including a putative TCF/LEF1 site at position -199, and a BRN2 site at position -48 (see later section). Transfected melanoma cells were treated for 48 hours with the indicated combination of aspirin and I3C and reporter gene activity compared to vehicle control treated cells. As shown in Figure 4b, treatment with either aspirin or I3C down regulated MITF-M promoter activity, demonstrating for the first time that aspirin and I3C signaling targets the MITF-M promoter. Furthermore, combinations of aspirin and I3C more significantly down regulate MITF-M promoter activity compared to the effects of either compound alone. This cooperative effect of combinations of aspirin and I3C suggest that each compound triggers distinct signaling pathways that target different regions of the MITF-M promoter, although relatively little is known about aspirin and I3C regulated signaling cascades that can potentially regulate MITF-M promoter activity.

Independent effects of Aspirin and I3C on production of Wnt/β-catenin signaling pathway components, Prostaglandin E_2 (PGE_2) levels and *in vitro* invasion properties of melanoma cells

Before further analyzing whether I3C and aspirin signaling acts through distinct regions of the MITF-M promoter, it was important to determine whether the cellular effects of each compound in melanoma cells are independent of each other. Aspirin has been shown to interfere with Wnt/β-catenin signaling in colorectal cancer cells by decreasing β-catenin protein stability and loss of beta-catenin regulated gene expression (Gala and Chan, 2015), suggesting the possibility that aspirin could be acting through this pathway in human melanoma cells. Because little is known about the effects of aspirin on Wnt/β-catenin signaling in melanoma cells, we examined the expression of several critical members of the Wnt/β-catenin signaling cascade in G361 melanoma cells treated for 48 hours with combinations of 3.0 mM aspirin and/or 200 μM I3C. Western blot analysis demonstrated that aspirin, but not I3C, strongly down regulated the protein levels of the LDL receptor-related protein 6 (LRP-6), a Wnt co-receptor, and of β-catenin (Figure 5a, left panels). Furthermore, aspirin up regulated Axin levels, which has been shown to act as a negative regulator of β-catenin stability (Clevers and Nusse, 2012). Densitometric quantification of the western blot results are shown in Figure 5b and confirm the selective effects of aspirin on regulating the levels of specific components of the Wnt signaling pathway (Figure 5b).

Analysis of Prostaglandin E_2 (PGE_2) levels and cell invasion properties suggest aspirin and I3C trigger distinct responses in human melanoma cells, and that the actions of these compounds do not significantly interfere with each other. PGE_2 levels can be regulated by a variety of factors in cancer cells (Kochel and Fulton, 2015), and it is well established that aspirin inhibits the Cyclooxygenase 1 and 2 (COX-1/2) enzymes that are responsible for the production of prostaglandins (Flower, 2003). To confirm aspirin was indeed acting as expected in the melanoma cells, the levels of PGE_2 were determined in G361 melanoma cells treated for 48 hours with combinations of aspirin and/or I3C. As shown in Figure 5c, even though I3C stimulated a modest increase in PGE_2 levels, aspirin strongly down regulated PGE_2 levels to approximately the same extent (~150 pg/ML) in the presence or absence of I3C. To assess another cellular process, *in vitro* cell invasion through the Matrigel membrane was examined in G361 melanoma cells treated with combinations of I3C and/or aspirin for approximately 22 hours. As shown in Figure 5d, treatment with I3C had a significantly greater inhibitory effect on in vitro cell invasion compared to aspirin (80% vs 20% inhibition), and the combination of both compounds displayed an apparent additive inhibitory effect. Taken together, these results indicate that I3C and aspirin mediate their cellular effects through distinct signaling pathways, and suggest that each compound requires different DNA elements in the MITF-M promoter to down regulate MITF-M promoter activity.

Aspirin and I3C disrupt MITF-M promoter activity through the LEF1 and BRN2 DNA binding sites

A mutational analysis was used to functionally test whether the combinational effects of aspirin and I3C on MITF-M promoter activity is due to each compound targeting distinct DNA elements in the MITF-M promoter. The -333 bp MITF promoter fragment within the

pGL2-333/+120-MITF-M reporter plasmid contains a consensus TCF/LEF1 DNA binding site at position −199 bp (CTTTGAT), and a BRN2 binding site at position −48 bp (CATAACTAATT) of the MITF-M promoter. TCF/LEF1 is a down-stream effector of Wnt signaling in that upon Wnt activation, β-catenin translocates to the nucleus and binds to the TCF/LEF family of transcription factors forming a complex that recruits co-activators to Wnt regulated genes (MacDonald et al, 2009, Mosimann et al, 2009, Takeda et al, 2000). The transcription factor BRN2 is down stream and activated by BRAF/MEK/ERK signaling (Goodall et al, 2004), and we recently observed that I3C disrupts BRN2 nuclear localization and binding to the MITF-M promoter through the inhibition of oncogenic BRAF signaling (Kundu et al, 2016). To test whether the LEF1 and BRN2 binding sites in the MITF-M promoter are required for the aspirin and I3C effects on MITF-M promoter activity, combinations of the LEF1 and/or BRN2 canonical DNA binding sites in the MITF-M promoter were mutagenized in pGL2-333/+120-MITF-M at base pairs known to be critical for transcription factor binding (Figure 6a). One mutant construct containing only mutated LEF1 site (ΔLEF1), another construct was mutated only in the BRN2 site (ΔBRN2) and a third construct was mutated in both the LEF1 and BRN2 sites (ΔLEF1/BRN2).

Each MITF-M promoter construct was linked to the luciferase reporter plasmid, transfected into either G361 or SK-MEL-30 melanoma cells and treated with combinations of 3.0 mM aspirin and/or 200 μM I3C for 48 hours. Reporter gene activity was measured and compared to vehicle control treated cells. As shown in Figures 6b and 6c, in both melanoma cell lines, combinations of aspirin and/or I3C treatment strongly down regulated MITF-M promoter activity. The cooperative effect of treatment with both compounds was particularly noticeable in the transfected G361 melanoma cells (Figure 6b, upper left panel). Mutation of the wild type LEF1 DNA binding site to CTTTGgcΔ aspirin down regulation of MITF-M promoter activity, whereas, the I3C down regulated response remained mostly intact in both cell lines (Figure 6b and 6c, lower left panels). In a complementary manner, mutation of the wild type BRN2 binding site to CATAACTAgcg (forming the ΔBRN2 reporter plasmid) disrupted the I3C down regulation of MITF-M promoter activity and did not alter the aspirin down regulation of MITF-M promoter activity (Figures 6b and 6c, upper right panels). Finally, mutation of both the LEF1 and BRN2 canonical DNA binding sites (forming the ΔΔBRN2 reporter plasmid) disrupted both the aspirin and I3C down regulation of MITF-M promoter activity (Figures 6b and 6c, lower right panels). Intriguingly, in G361 melanoma cells, treatment with aspirin, either alone or with I3C, had a mild stimulatory effect on MITF-M promoter activity that was observed only in the presence of the double mutated promoter. Taken together, our results demonstrate that the cooperative effects of aspirin and I3C on the down regulation of MITF-M promoter activity and gene expression can be accounted for by the selective targeting of distinct transcription factor binding sites in the MITF-M promoter.

Discussion

MITF-M, the “master regulator” of melanocytes, displays a complex link to melanoma progression and proliferation (Levy et al, 2006, Pierrat et al, 2012), and is considered a lineage addiction oncogene (Garraway et al, 2005) that is elevated in 10–20% primary melanoma tissue, and to an even higher degree in metastatic melanoma (Haq and Fisher,

2011). Furthermore, MITF-M expression is negatively correlated with five-year survival of this cancer (Garraway et al, 2005). A “rheostat model” has been proposed in which elevated expression levels of MITF-M lead to increased differentiation and cell cycle arrest, whereas, continued moderate levels of MITF-M expression signals melanoma cell survival and proliferation depending on the cellular mutation profile (Gray-Schopfer et al, 2007, Levy et al, 2006). It is also proposed that low to depleted levels of MITF-M expression is linked to quiescence or senescence, apoptosis and/or cell cycle arrest mediated by the loss of specific MITF-M target genes (Carreira et al, 2006, McGill et al, 2002). Therefore, the management of MITF-M levels and transcriptional function is considered to be a critical factor in determining effectiveness and treatment outcomes of a given melanoma therapy (Cirenajwis et al, 2015). Attenuation or enhancement of MITF-M promoter activity represents an important cellular mechanism to acutely control MITF-M gene expression. Intriguingly, the MITF-M promoter includes canonical sequences for the binding of variety of transcription factors (Drdova and Vachtenheim et al, 2004, Wellbrock et al, 2008), which implicate several different cell signaling pathways that can potentially target MITF-M promoter activity and are potential targets of therapeutic strategies. However, relatively little is known about how potential anti-melanoma compounds influence the transcriptional control of MITF-M promoter activity.

Our studies have established that the cooperative anti-proliferative effects of aspirin and I3C in human melanoma cells trigger a significant down regulation of MITF-M gene expression and disruption of MITF-M promoter activity. The aspirin and I3C induced G1 cell cycle arrest likely results from the loss of MITF-M expression because several essential G1 acting cell cycle regulators that are down regulated in melanoma cells, such as CDK2 and CDK4, are MITF-M target genes (Du et al, 2004, Wellbrock et al, 2008). Aspirin and I3C targeted signaling pathways converge on the MITF-M promoter at distinct transcription factor binding sites in that the aspirin mediated portion of the response is dependent on the LEF1 binding site and the I3C effect is dependent on the BRN2 binding site. The LEF1/TCF family of transcription factors is a down stream effector of the canonical Wnt signaling pathway that forms a complex with nuclear localized β-catenin to promote MITF-M gene expression (Takeda et al, 2000). Consistent with this concept, we have observed that aspirin disrupts Wnt signaling in melanoma cells by down regulating levels of the LRP-6 Wnt co-receptor protein and β-catenin as well as by elevating expression the Axin component of the destruction complex that can ubiquitin and target β-catenin for its proteasome mediated degradation. We propose that the aspirin-dependent loss of β-catenin protein prevents its interaction with LEF1 on the MITF-M promoter.

The canonical Wnt/β-catenin signaling pathway in non-transformed cells is essential for cell processes such as maintaining cell polarity, movement, proliferation, differentiation, and survival. In many malignancies, an activating mutation in Wnt/β-catenin signaling occurs, which typically allows for inhibited activity of the destruction complex responsible for tagging cytoplasmic β-catenin for proteasomal destruction (Bjorklund et al, 2009, Polakis, 2007). The stabilized β-catenin translocates to the nucleus and binds with the TCF/LEF family of transcription factors, allowing for activation of downstream genes. Activating mutations are common in colorectal cancers as well as one third of melanomas, with that subset demonstrating significantly poorer prognosis than those without aberrant Wnt

signaling. In both types of cancer, activation of the Wnt/β-catenin pathway is an essential driver to cancer progression and chemoresistance (Chikazawa et al, 2010, Sinnberg et al, 2011). In metastatic melanoma cell lines, blocking β-catenin expression has been demonstrated to induce apoptosis, inhibit proliferation, and migration (Vaid et al, 2011, You et al, 2004). In addition, it has recently been demonstrated that melanomas with active β-catenin signaling is associated with the absence of T-cell infiltration in the tumor microenvironment and inhibited host immune response, even blocking the activities of current immunotherapeutics (Spranger et al, 2015).

Our results demonstrate for the first time that aspirin modulates the canonical Wnt signaling pathway in melanoma cells, although previous work has shown that aspirin interferes with Wnt/β-catenin signaling in colorectal cancer by increasing β-catenin phosphorylation at Ser33 and Ser37 decreased activity of protein phosphatase 2A (PP2A), providing a recognition site for the destruction complex and as a consequence, a decrease in β-catenin stability (Bos et al, 2006). The identification of aspirin as a cancer preventative agent emerged from the significant reductions in colorectal cancer and melanoma rates and mortality after a 5-year period in individuals who ingested daily doses of aspirin for cardiovascular problems (Flossmann and Rothwell et al, 2007). While low dose aspirin (81mg/day) is deemed relatively safe, higher doses (325mg/day or higher) such those used to treat sympotoms of inflamation and pain have the potential side effect of preventing clotting and increase the risk for gastric bleeding. As a result, strategies to decrease blood concentration of aspirin and its metabolites while maintaing its anti-cancer effects would be advantageous in a clinical setting. We have demonsrated that the *in vitro* concentrations of aspirin can be reduced to achive anti-proliferative effects in human melanoma cells by combining it with I3C, an anti-cancer phytochemical with few side-effects in humans (Reed et al, 2005). In contrast to aspirin, I3C required the BRN2 transcription factor site in the MITF-M promoter to mediate its portion of the combined effect on MITF-M gene expression and had relatively little effect on Wnt signaling or other aspirin responses, such as PGE2 production. Conversely, I3C had a significantly stronger effect on melanoma cell invasion compared to aspirin. We have recently observed that I3C disrupts down stream BRAF signaling resulting in the loss of BRN2 binding to and activating the MITF-M promoter (Kundu et al, 2016), which suggests that aspirin and I3C target the MITF-M promoter through distinct cell signaling cascades.

In Phase I and II clinical trials adult oral doses of I3C as high as 800mg/day have been shown to be well tolerated and lacking significant toxicity in humans (Reed et al, 2005). Pharmacokinetic studies with animal models show that I3C and its bioactive acid condensation products DIM, indolo[3,2-b]carbazole, and 1-(3-hydroxymethyl)indolyl-3-indolylmethane are mostly absorbed from the plasma within 1 h and a mixture of indole compounds, including I3C, accumulate in a variety of indole-sensitive tissues with the highest level in the liver (Aggarwal and Ichikawa, 2005). Despite the relative low plasma levels of indoles observed after I3C is taken orally, the accumulated tissue levels of indoles are high enough to alter estrogen metabolism, suggesting that the organ-attainable level of specific indoles can account for their overall *in vivo* anticancer effects. Consistent with our observations with human melanoma cells, in cell culture studies using several different types of human cancer cells, generally the maximal anti-proliferative effects were observed in the

100μM to 250μM concentration range of I3C (Ahmad et al, 2012, Firestone and Sundar, 2009, Sarkar et al, 2009, Aggarwal and Ichikawa, 2005). One reason for the need of relatively high concentrations of I3C in cell culture is that even though I3C displays a long half-life in cell medium, the effective cellular concentration is much lower because only approximately 0.3% of this compound enters the cell and available to react with or bind to specific targets (Staub et al, 2002).

Combined cancer therapies have several advantages over the use of treatments with individual compounds. Treatments with combinations of anti-cancer drugs that act through different mechanisms can reduce the likelyhood of reisistant tumor cells escaping treatment and developing into a new resistant tumor. Furthermore, because of cooperative effects, a smaller dose of each drug may be given to achieve similar results, which can potentially reduce unintended side effects of higher drug concentrations. To date, no studies have assessed the effectiveness of either I3C or aspirin directly on skin, which may be a more effective way to treat melanomas and should significantly enhance the half-life of the compounds by avoiding the acidic conditions of the stomach. Given the high tolerance for I3C in humans, and the numerous clinical studies analyzing the tolerance of both low dose and high dose aspirin, the cooperative effects between I3C and aspirin demonstrated in our study provide evidence that this drug combination of realtively well tolerated phytochemicals has potential as an effective anti-melanoma adjuvant therapy or perhaps as a preventative treatment. Also, the combination aspirin and I3C display potent anti-proliferative effects in melanoma cells expressing either a wild type or oncogenic form of BRAF, suggesting that a range of melanomas with distinct mutation profiles will potentially be sensitive to combinations of aspirin and I3C as a therapeutic strategy.

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Abbreviations

BRN2	Brain-2
COX	Cyclooxygenase
LEF1	Lymphoid Enhancer-binding Factor 1
I3C	Indole-3-carbinol
MITF-M	Microphthalmia Associated Transcription Factor isoform M
NEDD4-1	Neural precursor cell Expressed Developmentally Down-regulated 4

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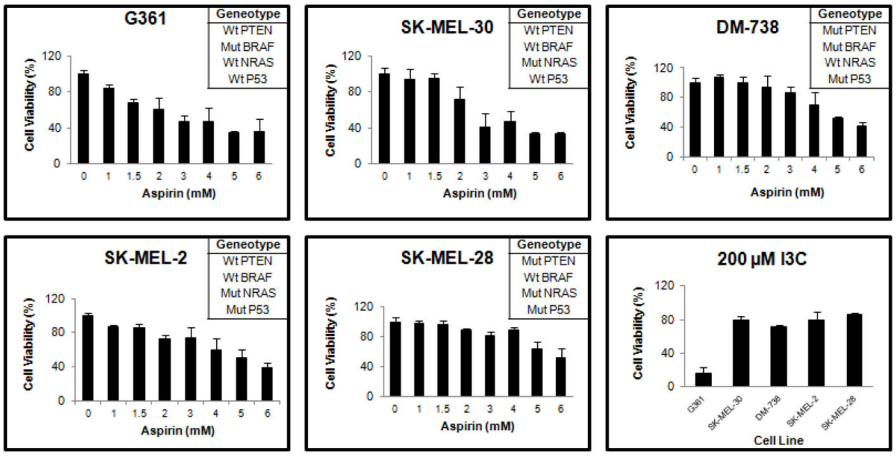

Fig 1.

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

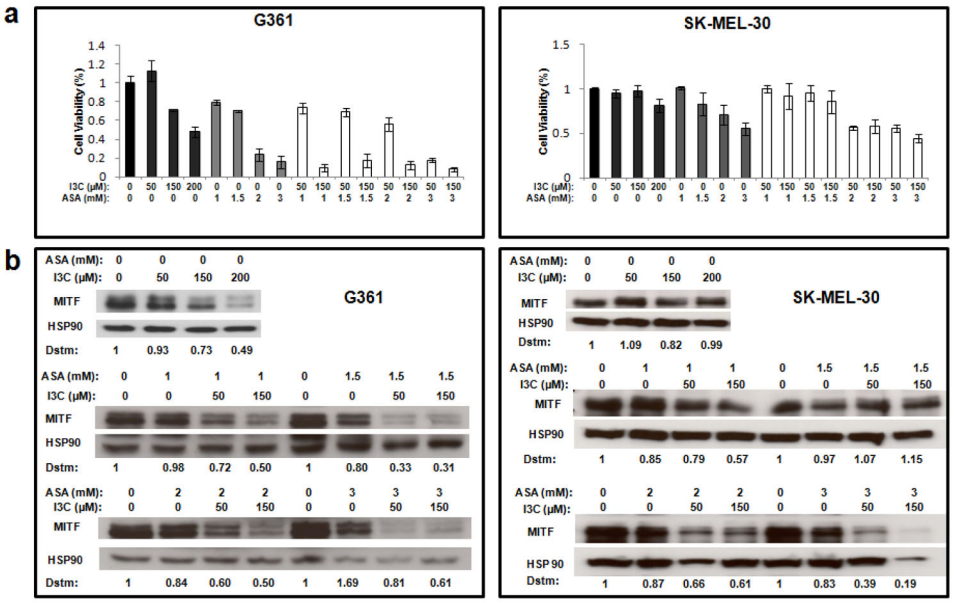

Fig 2.

Combinational effects of aspirin and I3C on melanoma cell proliferation and production of MITF-M protein. (a) G361 cells (left panel) and SK-MEL-30 cells (right panel) were treated with the indicated concentrations of aspirin (ASA) or I3C for 48 hours and cell proliferation was measured using a CCK-8 assay relative to vehicle control treated cells. (b) G361 cells (left panel) and SK-MEL-30 cells (right panel) were treated with the indicated concentrations of aspirin (ASA) or I3C for 48 hours and the levels of MITF-M protein were analyzed by western blots. The relative levels of MITF-M protein under each condition were measured by densitometry (Dstm) of the western blots and normalized to the HSP90 loading control protein.

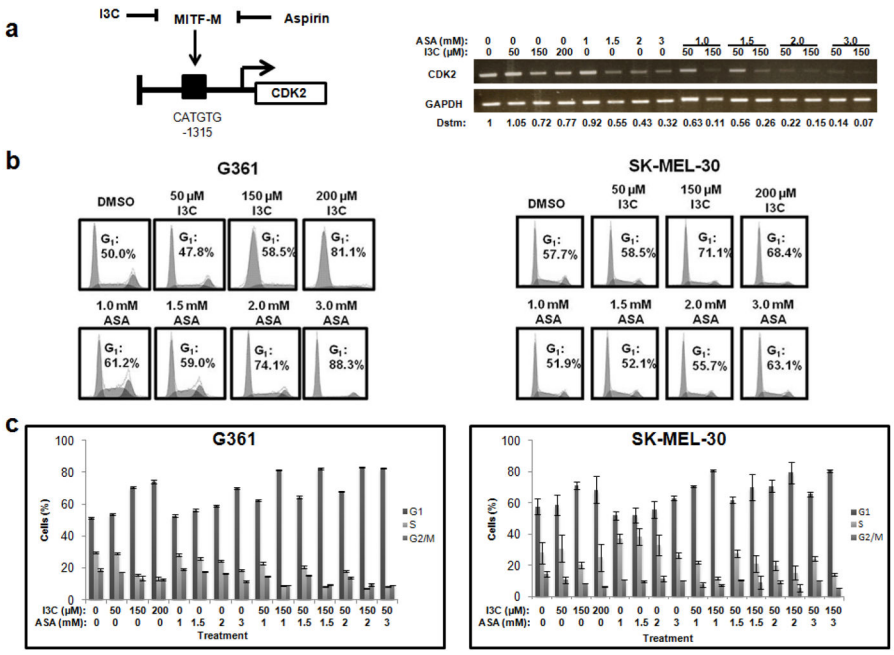

Fig 3.

Aspirin and I3C regulation of melanoma cell cycle progression and expression of CDK2. (a) The diagram describes the MITF-M interaction with its DNA binding site in the CDK2 promoter. CDK2 transcript expression in G361 cells treated with the indicated concentrations of aspirin (ASA) and I3C was determined by RT-PCR analysis in comparison to the GAPDH control transcript. Densitometry (Dstm) was utilized to measure relative intensity of the expressed CDK2 transcripts in each condition normalized to the ratio of CDK2:GAPDH in vehicle control treated cells. (b) Flow cytometry profiles of G361 cells (left panels) or SK-MEL-30 cells (right panels) treated with either aspirin (ASA) I3C for 48 hours. The average percent of the cell population in the G_1 phase from three independent experiments is shown for each representative flow cytometry profile. (c) The effects of the indicated combinations of ASA and I3C concentrations on G361 (left panel) and SK-MEL-30 (right panel) cell cycle after 48 hours treatment was determined by flow cytometry. The values represent the average of three independent experiments.

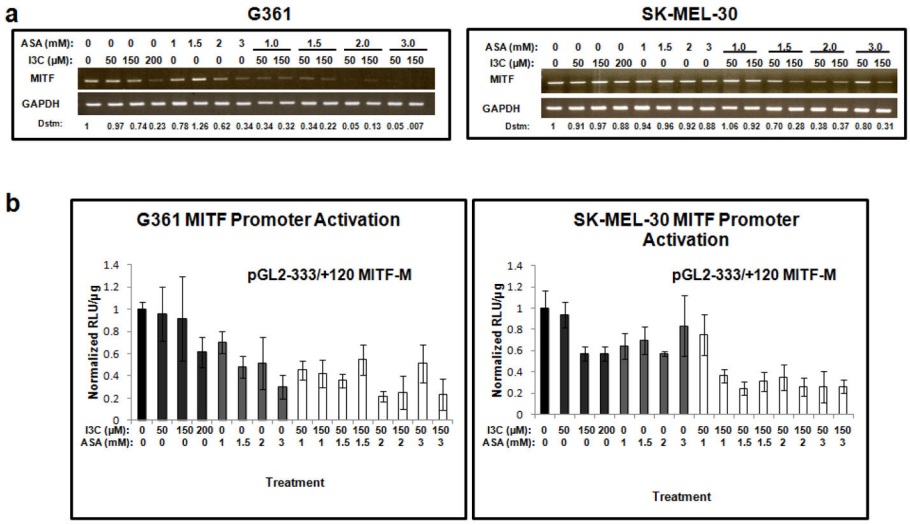

Fig 4.

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

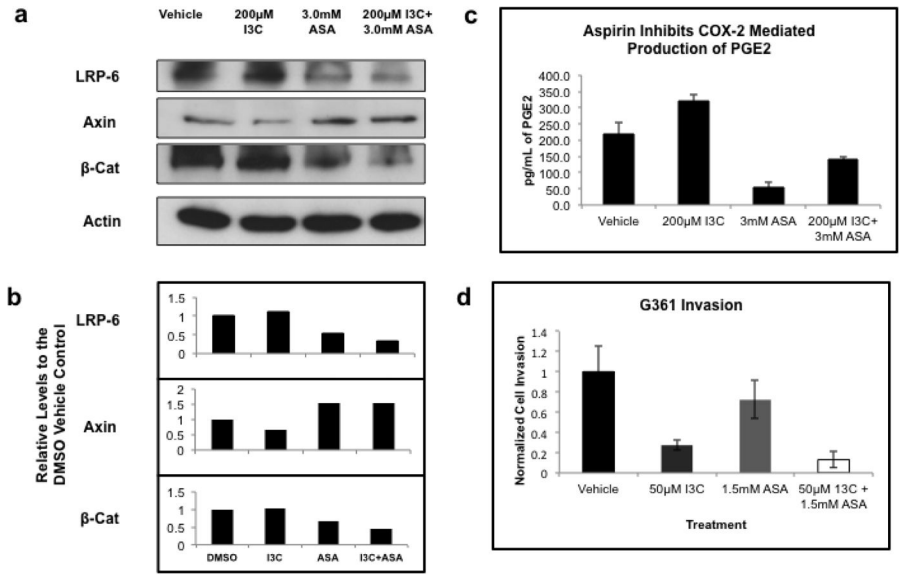

Fig 5.

Independent effects of I3C and aspirin on production of Wnt signaling components, PGE2 levels and *in vitro* invasion properties. (a) G361 melanoma cells were treated with the indicated combinations of 200 μM I3C and/or 3.0 mM aspirin (ASA) or with the DMSO vehicle control for 48 hours. Western blots of electrophoretically fractionated cell extracts were probed for LRP-6, Axin, and β-catenin protein or for the actin gel loading control protein. (b) Densitometry analysis to quantify LRP-6, Axin and β-catenin protein levels relative to actin protein that was observed in the western blot (c) The production of PGE2 was determined as described in the methods and material section and averages of results from three independent experiments are shown in the bar graphs. (d) The effects of combinations of 50μM I3C and/or 1.5mM aspirin on the in vitro invasion properties of G361 melanoma cells were determined as described in the methods and materials section. The bar graphs represent the average triplicate results from three independent experiments.

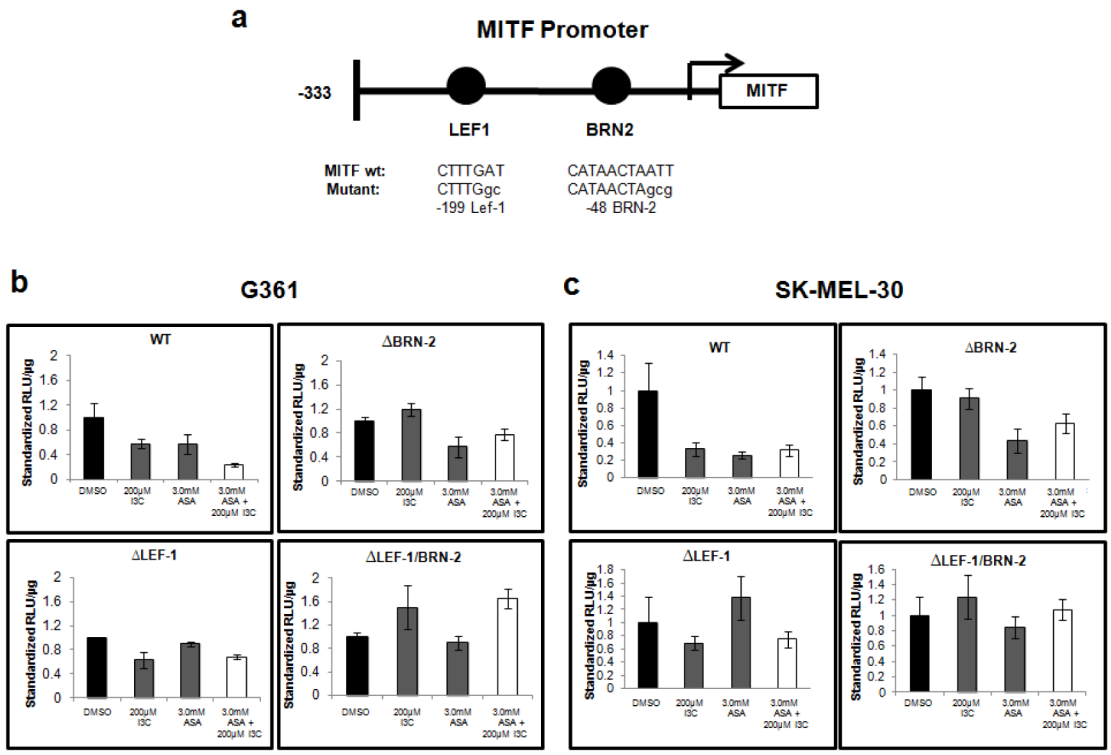

Fig 6.

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