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3,3'-Diindolylmethane (DIM) Inhibits the Growth and Invasion of Drug-Resistant Human Cancer Cells Expressing EGFR Mutants

Massod Rahimi^a, Kai-Ling Huang^a, and Careen K. Tang^{a,*}

^aLombardi Comprehensive Cancer Center, Department of Oncology, Georgetown University Medical Center, Washington, DC 20057

Abstract

Epidermal Growth Factor Receptor (EGFR) mutants are associated with resistance to chemotherapy, radiation, and targeted therapies. Here we found that the phytochemical 3,3'-Diindolylmethane (DIM) can inhibit the growth and also the invasion of breast cancer, glioma, and non-small cell lung cancer cells regardless of which EGFR mutant is expressed and the drug-resistant phenotype. DIM reduced an array of growth factor signaling pathways and altered cell cycle regulators and apoptotic proteins favoring cell cycle arrest and apoptosis. Therefore, DIM may be used in treatment regimens to inhibit cancer cell growth and invasion, and potentially overcome EGFR mutant-associated drug resistance.

Keywords

EGFR; Mutants; Drug-Resistance; Growth; Invasion; DIM

1. Introduction

The Epidermal Growth Factor Receptor (EGFR) is often associated with aggressive disease, metastasis, and drug resistance in human cancers [1]. In breast cancer, high EGFR expression is associated with poor prognosis and resistance to endocrine therapy and chemotherapy [1-3]. Over 50% of gliomas have EGFR gene amplification, and a majority of these cases also express the EGFR type III mutation (EGFRvIII) [4;5]. EGFRvIII is associated with radioresistance as well as chemoresistance [1;4;5]. Finally, non-small cell lung cancer patients with high EGFR expression are often treated with tyrosine kinase inhibitors (TKIs). A number of mutations in EGFR have been identified in the tumors of these patients, where the in-frame deletion delE746-A750 or the L858R amino acid substitution leads to increased sensitivity, while the T790M amino acid substitution confers resistance to TKIs [6-9]. Therefore, exploration of alternative treatment strategies for EGFR mutant-associated drug resistance is warranted. New antitumor compounds which are well tolerated by cancer patients are required to target EGFR as a first-line treatment regimen or second-line treatment after the emergence of resistance to pre-existing anti-EGFR agents.

*Corresponding author: E512 The Research Building, 3970 Reservoir Road, NW, Washington DC 20057, Phone: (202) 687-0361, Fax: (202) 687-7505, Tangc@georgetown.edu.

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Phytochemicals have been identified as potential chemopreventive agents as these natural compounds have been shown to have antitumor effects in preclinical models and humans [10;11]. One of these compounds which is actively being pursued for human usage is 3,3'-Diindolylmethane (DIM), produced from the digestion of Indole-2-Carbinol (I3C) in cruciferous vegetables. DIM has been shown to reduce carcinogen-induced breast and lung tumor formation in rodent models of carcinogenesis with little toxicity [12-16]. Neovascularization and human breast tumor xenografts were also shown to be inhibited by DIM administration [17]. Furthermore, DIM in combination with taxanes has been shown to inhibit breast and lung cancer cell growth *in vitro* as well as *in vivo* [13;18;19].

The mechanism by which DIM inhibits cancer cell growth and induces apoptosis is actively being studied, although results remain inconsistent. DIM induces cell cycle arrest by inducing the expression of cell cycle inhibitors such as p21 and p27 or by reducing the expression of cyclins such as Cyclin D1 or cyclin dependent kinases [16;20-25]. DIM also induces apoptosis in breast and lung cancer cells by down-regulating Survivin and Bcl-2, decreasing Bax, inducing poly (ADP-Ribose) polymerase (PARP) cleavage, releasing mitochondrial cytochrome c, and enhancing procaspase cleavage [13;18;22-24;26]. Down-regulation of estrogen receptor (ER)-alpha expression through the activation of the aryl hydrocarbon receptor (AhR), activation of interferon gamma (IFNgamma), increases in major histocompatibility complex class-I (MHC-1) molecules, and activation of the peroxisome proliferators-activated receptor gamma (PPARGgamma) have each been shown to be essential for DIM's antitumorigenic properties in breast cancer [16;27-29].

DIM has been shown to have profound effects on ErbB-receptor and downstream signaling pathways. In ErbB2-expressing breast cancer, DIM has been shown to induce cell cycle arrest and apoptosis by suppressing the activation of ErbB2 and downstream signaling cascades including Akt and NF-kappaB, which are downstream of growth factor signaling [18;19]. DIM was also shown to induce apoptosis in MCF10A derived malignant cells, but not in non-tumorigenic parental MCF10A cells by inhibiting EGF-induced activation of Akt and NF-KappaB [30]. Erlotinib and B-DIM (a formulated DIM with greater bioavailability) reduced cell viability, induced apoptosis, down-regulated EGFR phosphorylation, NF-kappaB DNA-binding activity, and expression of anti-apoptotic genes in pancreatic cells [31]. DIM can also down-regulate EGFR expression and inhibit PI3K/Akt and NF-KappaB activity in prostate cancer cells [32]. Although I3C can reduce EGFR levels in breast cancer cells [33], the ability of DIM to inhibit the expression or activity of EGFR mutants in cancer cells of the breast, lung, and central nervous system has never been explored. Furthermore, whether DIM treatment can overcome EGFR mutant-associated drug resistance in human cancers has never been evaluated.

In this study, we investigated the ability of DIM to target tamoxifen or radiation/chemotherapy resistance-associated EGFR mutant, EGFRvIII, found in breast cancer and gliomas, and the TKI-resistant tyrosine kinase domain mutants of EGFR in non-small cell lung cancers. Regardless of which mutant form of EGFR was expressed in the cells, the growth of all these drug-resistant EGFR mutant-expressing cell lines was sufficiently inhibited by DIM. Both the phosphorylation and protein levels of EGFRvIII in breast cancer and glioma cells, as well as mutant EGFRs found in H1650 (delE746-A750) and TKI-resistant H1975 (L858R+T790M) non-small cell cancer cells, were significantly suppressed by DIM. DIM also inhibited the activation and protein levels of other growth factor receptors. Interestingly, DIM-mediated inhibition of proliferation through abrogation of downstream signaling pathways of growth factor receptors as well as alterations in molecules involved in cell cycle and apoptosis were varied in different cell lines. The changes in protein levels were often associated with cell cycle arrest and increased apoptosis. Furthermore, DIM also reduced the invasive potential of cells expressing EGFRvIII in breast cancer and glioma cells as well as the mutations of EGFR in the lung cancer cells.

2. Materials and Methods

2.1. Cell Culture and Reagents

Human breast cancer (MDA-MB-361 and MCF-7), glioma (H4 and U87MG), and non-small cell lung cancer (H1650 and H1975) cell lines were maintained in IMEM, DMEM, or RPMI-1650 (MediaTech) supplemented with fetal bovine serum (FBS), L-glutamine, and sodium pyruvate where appropriate. Stable EGFRvIII-expressing cells were generated as previously described [34]. 3,3'-Diindolylmethane (DIM) was purchased from LKT Laboratories (St. Paul, MN).

2.2. Growth assays

Cancer cells were seeded in triplicates in 24-well plates. Using a cell counter, cancer cells were counted on days 0, 1, 2, and 3 after being treated with vehicle (DMSO), 10 and 20 μ M DIM (breast), 20 and 30 μ M DIM (glioma), or 20 and 50 μ M DIM (lung). Percent of control was calculated by dividing number of cancer cells treated with DIM/number of cancer cells treated with vehicle.

2.3. Immunoblot analysis

Cancer cells were plated in culture plates and grown to 50% to 60% confluence, and then treated with vehicle (DMSO) or 20 μ M (breast), 30 μ M (glioma), or 50 μ M (lung) DIM for 48 hours (in growth media). After the removal of the media, cells were rinsed, and then lysed in a lysis buffer containing 50 mM HEPES, 1% Triton X-100, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4 , 1 mM PMSF, 20 μ g/mL leupeptin, 10 μ g/mL aprotinin, 100 mM NaF, and 10 μ g/mL of trypsin inhibitor. Cell extracts were clarified by centrifugation at 13,000 rpm, protein concentration was determined by BCA Protein Assay (Pierce, IL, USA), and equal amounts of protein were then separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare Life Sciences; Piscataway, NJ) for immunoblot analysis. Membranes were blocked with TBST containing 5% non-fat dry milk for 1 hour at room temperature with constant agitation. Membranes were probed with appropriate primary antibody for 2 hours at room temperature or overnight at 4°C, washed three times with TBST, and then incubated with secondary antibody for 1 hour at room temperature, and finally, washed three times with TBST. Immunoreactive bands were visualized by a chemiluminescence reagent (Pierce; Rockford, IL), and exposed to film. The antibodies against phospho-EGFR (Tyr1148, Tyr992, or Tyr1173), phospho-ErbB2 (Tyr877), phospho-Met (Tyr1234/1235), phospho-Akt (Ser473), phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, p21, p27, Cyclin D1, CDK6, CDK4, Bad, Bax, Bcl-2, and Bcl-xL were purchased from Cell Signaling Technology (Beverly, MA); the antibodies for EGFR and ErbB2 were purchased from Neomarkers (Fremont, CA); antibodies against Met, Akt (Akt1), PARP, and Survivin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and the antibody for GAPDH was purchased from Sigma-Aldrich (St. Louis, MO). The secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse (GE Healthcare Life Sciences; Piscataway, NJ), donkey anti-rabbit (GE Healthcare Life Sciences; Piscataway, NJ), or donkey anti-goat (Santa Cruz Biotechnology, Inc.).

2.4. Invasion assays

Serum-induced invasion was measured using 24-well cell culture inserts with membranes with 8 μ m pores and a matrigel-coating (BD Biosciences; San Jose, CA). Cancer cells were suspended in serum-free medium with 0.1% BSA and plated in the top part of the insert. The vehicle (DMSO) or 10 μ M (breast and glioma) or 20 μ M (lung) DIM was added as pre-treatments and to the cell suspension. The inserts were placed in wells containing media supplemented with FBS. After incubation at 37°C for 48 hours, residual cells were wiped off

the top of the membranes with cotton swabs, and invaded cells on the underside of the membranes were fixed and stained using the HEMA-3 kit (Fisher Diagnostics, Middletown, VA). Cells were counted in 10 fields from three inserts per experimental condition.

2.5. Statistics

Statistical analysis was performed using ANOVA, followed by the Tukey test using SigmaStat software (Systat Software; San Jose, CA). Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. DIM inhibits the growth of drug resistant EGFR mutant-expressing cancer cells

Previous reports have shown that DIM inhibits the growth of various cancer cells [13;16-19; 21-26;30]. To determine if DIM also inhibits the growth of cancer cells expressing EGFR mutants associated with drug resistance (See Table 1 for a summary of the drug-resistant phenotypes associated with EGFR mutants), we performed *in vitro* anchorage-dependent cell growth assays on six different cancer cell lines from three different origin of cancers expressing either EGFRvIII or tyrosine kinase mutants of EGFR. DIM inhibited the growth of both breast cancer and glioma cells expressing EGFRvIII, as well as non-small cell lung cancer cells expressing the delE746-A750 (H1650 cells) and TKI-resistant L858R+T790M (H1975 cells) EGFR mutations (Fig. 1). In general, breast cancer cells were more sensitive to DIM inhibition with the lowest IC₅₀ (10 and 20 μ M), while glioma cells required slightly higher concentrations (20 and 30 μ M), and lung cancer cells were less sensitive with the highest IC₅₀ (20 and 50 μ M). Interestingly, MCF-7 breast cancer cells, which express low levels of ErbB2, were less sensitive to the growth inhibitory effects of DIM than were high ErbB2-expressing MDA-MB-361 breast cancer cells (Fig. 1A). Noteworthy, MDA-MB-361 breast cancer cells expressing EGFRvIII are estrogen-independent and tamoxifen-resistant, whereas MCF-7 breast cancer cells expressing EGFRvIII remain estrogen-dependent and tamoxifen-sensitive *in vivo* [35]. However, ectopic EGFRvIII expression in these breast cancer cells rendered the cells less sensitive to DIM treatment in comparison to that of parental cells regardless of tamoxifen-sensitivity (Fig. 1A). In contrast, glioma cells expressing EGFRvIII exhibited similar sensitivity to DIM inhibition in comparison to control cells (Fig. 1B). The TKI-sensitive H1650 lung cancer cells were also more sensitive to the growth inhibition induced by DIM in comparison to that of TKI-resistant H1975 lung cancer cells (Fig. 1C). Nevertheless, these results suggest that DIM treatments may be able to circumvent tamoxifen, radiation/chemotherapy, and TKI resistance in breast, glioma, and non-small cell lung cancer cells expressing EGFR mutants.

3.2. DIM decreases the phosphorylation and expression of receptor tyrosine kinases in cancer cells expressing mutated EGFR

Although DIM has also been shown to inhibit EGFR and ErbB2 expression/activity [18;19; 30-32], DIM suppression of mutated EGFR expression or activity has never been shown. Our results revealed that DIM not only inhibits the phosphorylation of EGFRvIII, it also decreases EGFRvIII expression in breast and glioma cells (Fig. 2A and B). Surprisingly, H1650 and H1975 lung cancer cells also have decreased EGFR phosphorylation, but a much less pronounced effect on the reduction of EGFR expression was observed in these cells (Fig. 2C). Since EGFR and EGFR mutants heterodimerize with other ErbB-receptors and often crosstalk with other receptor tyrosine kinases, we wondered whether DIM also has inhibitory effects on other ErbB-receptors and other receptor tyrosine kinases. Amplification of ErbB2 has played a crucial role in breast cancer and Met has been implicated in the progression of a wide variety of cancers, including glioma and lung cancer [36-38]. The amplification of Met has recently been linked to TKI-resistant lung cancer [39;40]. We found that DIM also reduces the

phosphorylation and expression of ErbB2 in breast cancer cells (Fig. 2A) and inhibits the phosphorylation and expression of Met in glioma and lung cancer cells (Fig. 2B and C). Furthermore, full-length wild-type EGFR is also down-regulated in breast cancer and glioma cells, although the effect is more pronounced in glioma cells, which often express higher levels of full-length EGFR (Fig. 2A and B). These results confirm that DIM inhibits the growth of cancer cells by targeting growth factor receptors.

3.3. DIM decreases the activation of survival/proliferative signaling pathways, while increasing the activation of stress-activated signaling pathways

DIM has also been shown to inhibit the activation of PI3K/Akt and p44/42 MAPK (ERK1/2) alone or in combination with other antitumor agents such as erlotinib or paclitaxel [18;19;30;32]. To determine if DIM inhibits downstream signaling cascades often activated in cancer cells expressing mutated EGFR, we evaluated the activation of Akt, p44/42 MAPK, and p38 MAPK upon DIM treatment. DIM was shown to universally reduce the activation and protein levels of Akt in all cell lines expressing EGFR mutants (Fig. 3), while p44/42 MAPK activation and protein levels were decreased in breast cancer cells expressing EGFRvIII (Fig. 3A) as well as lung cancer cells expressing mutated EGFR (Fig. 3C). In contrast, glioma cells either had unchanged or increased activation of p44/42 MAPK upon DIM treatments (Fig. 3B), suggesting a possible feedback mechanism between the survival and proliferative pathways involving Akt and MAPKs. DIM-treated cells also exhibited increased activation of p38 MAPK (Fig. 3), which is expected as activation of this stress pathway has been shown to inhibit cell growth and induce apoptosis in DIM-treated cells [29;41;42].

3.4. DIM alters the expression of cell cycle regulators and pro- and anti-apoptotic proteins, favoring cell cycle arrest and apoptosis

Early studies have shown that DIM inhibits cancer cell growth by inducing cell cycle arrest and/or apoptosis [13;16-19;21-26;29;30;41;42]. Interestingly, DIM-mediated growth suppression in cancer cells expressing EGFR mutants seems to occur through differential modulation of cell cycle regulators. We found that some cancer cells had either increased expression of the cell cycle inhibitors, p21 and p27, decreased expression of cell cycle drivers such as Cyclin D1, CDK4, and CDK6, or combinations all suggesting cell cycle arrest due to heterogeneity of tumor cells (Fig. 4). In breast cancer model systems regardless of EGFRvIII expression, all cell lines have an enhanced expression of p21 or p27, and a reduction in Cyclin D1, CDK4, and CDK6 expression upon DIM treatment (Fig. 4A). In glioma model systems, although we did not observe down-regulation of Cyclin D1 in H4 cells by DIM treatment possibly due to p16 deficiency in these cells, the expression of CDK4 and CDK6 was reduced and either p21 or p27 expression was up-regulated (Fig. 4B). In lung cancer model systems, DIM decreased Cyclin D1 as well as CDK4 and CDK6 in both H1650 and H1975 cells (Fig. 4C). The cell cycle inhibitor p27 remains unchanged in both lung cancer cell lines, while p21 is up-regulated in H1975 cells by DIM (Fig. 4C).

We also used immunoblot analysis to determine which proteins involved in apoptosis are altered by DIM in EGFR mutant-expressing cancer cells. We found that DIM induces cleavage of PARP1, a hallmark of apoptotic cells, in several of the cell lines (Fig. 5). However, PARP1 cleavage was not associated with EGFRvIII expression, sensitivity to tamoxifen or TKIs, or sensitivity to the growth inhibitory effects of DIM (Fig. 1). We did observe a universal reduction in PARP2 expression in all the cell lines treated with DIM (Fig. 5). Furthermore, we found a reduction of the anti-apoptotic Survivin expression in all the cell lines treated with DIM (Fig. 5). No significant changes of pro- and anti-apoptotic proteins occurred in MCF-7 breast cancer cells, while MDA-MB-361 breast cancer cells had a reduction in the pro-apoptotic Bax and the anti-apoptotic Bcl-xL, which suggests a balancing of pro- and anti-apoptotic proteins within these cells (Fig. 5A). In glioma cells, DIM increased the expression of the pro-

apoptotic Bad and Bax, while the anti-apoptotic Bcl-2 was increased slightly, and the anti-apoptotic Bcl-xL was decreased (Fig. 5B). In lung cancer cells, Bcl-xL and Bax were unchanged by DIM treatment, while Bad expression was increased, and Bcl-2 expression was decreased (Fig. 5C). These results suggest that DIM induces apoptosis through PARP1 cleavage and alters the expression of apoptotic proteins leading to increased apoptosis in cancer cells.

3.5. DIM abolishes the invasive potential of cancer cells expressing mutated EGFR

Expression of EGFRvIII leads to increased invasiveness of cancer cells and is often associated with metastasis in humans [43-47]. We wondered whether DIM had the ability to inhibit the invasive potential of EGFRvIII-expressing breast cancer and glioma cells. Indeed, DIM inhibited the number of invading breast cancer and glioma cells expressing EGFRvIII in *in vitro* invasion assays (Fig. 6A and B). DIM also significantly reduced the invasiveness of H4 glioma cells that did not express EGFRvIII (Fig. 6B). In addition, we also discovered that the invasive potential of both H1650 and H1975 lung cancer cells was reduced by DIM (Fig. 6C). Therefore, in addition to inhibiting growth, the invasive potential of aggressive breast cancer, lung cancer, and glioma cells can also be inhibited by DIM.

4. Discussion

The rationale for using EGFR-targeted approaches for cancer treatment is now firmly established and numerous clinical trials are in progress. However, resistance to EGFR-targeted therapy universally emerges over time. The next challenge is to develop novel treatment strategies to overcome the intrinsic and acquired resistance. Some natural products such as the food derivatives I3C and DIM have antitumor properties and can prevent carcinogen-induced tumorigenesis and human tumor cell xenograft development in animal models as well as inhibit cancer cell growth in ErbB-receptor expressing cancer cells [12-19;25;31-33]. We investigated the inhibitory effects of DIM on human cancer cells of the breast, lung, and central nervous system (glioma) which express mutant forms of the EGFR. Our results report, for the first time, that although the breast and glioma cells expressing EGFRvIII or non-small cell lung cancer cells expressing EGFR mutations can lead to resistance to targeted therapies, and in some cases, chemotherapy or radiation, these cells are sensitive to the growth inhibition induced by DIM.

EGFR mutants can heterodimerize with other oncoproteins and crosstalk with other receptor tyrosine kinases and activate multiple downstream signaling networks. This crosstalk can occur either through a direct association between receptors, or indirectly, via common interaction partners or downstream signaling molecules. This multi-layered crosstalk can result in the induction of resistance to targeted therapies. In breast cancer, EGFRvIII is frequently co-expressed with ErbB2, and defective degradation pathways of EGFRvIII can lead to prolonged and enhanced EGFRvIII/ErbB2 signaling [47;48]. Although EGFRvIII exhibits defective degradation, we found that EGFRvIII was significantly down-regulated in breast cancer cells by DIM. Furthermore, high ErbB2-expressing, estrogen-independent, tamoxifen-resistant MDA-MB-361 breast cancer cells expressing EGFRvIII were much more sensitive to the growth inhibitory effects of DIM in comparison to low ErbB2-expressing, estrogen-dependent, tamoxifen-sensitive MCF-7 breast cancer cells expressing EGFRvIII [35]. The reduction of EGFRvIII and ErbB2 expression in MCF-7 breast cancer cells was also much less pronounced than that of in MDA-MB-361 breast cancer cells expressing EGFRvIII, suggesting that DIM treatment may overcome EGFRvIII-mediated tamoxifen resistance in breast cancer. Targeting the expression of EGFRvIII and ErbB2 protein, but not its activity may be of clinical benefit to breast cancer patients expressing EGFRvIII.

The expression of Met has been implicated in the progression of a wide variety of cancers, including glioma, lung, and breast cancer [36;37]. Amplification of Met and HGF has been

associated with highly invasive and metastatic tumors and poor patient prognosis in lung cancer and amplification of Met has recently been linked to TKI-resistant lung cancer [39;40]. Furthermore, the activity and expression of Met has been suggested to crosstalk with EGFR and synergize with EGFRvIII [5;49;50]. Our study demonstrates, for the first time, that DIM can simultaneously reduce the expression levels of Met and EGFR mutants in glioma and lung cancer cells. Hence, as a single agent, DIM can eliminate the oncogenes that drive tumorigenesis, and DIM is also able to prevent “oncogene switching” by targeting various oncogenic receptor tyrosine kinases simultaneously. Moreover, DIM may circumvent TKI resistance in EGFR mutant-expressing non-small cell lung cancer cells and radiation resistance in EGFRvIII-expressing glioma cells. These results suggest that DIM may be used in combination with conventional therapeutics for the treatment of human malignancies expressing EGFR mutants.

The complexity of signaling networks that develop in cancer cells frequently results in a redundancy and overlap of cell survival and proliferation pathways, potentially allowing cancer cells to circumvent the therapeutic effects of targeting one single growth or survival signaling pathway. Since the ErbB-receptor signaling network is enormously diversified, cancer cells exhibit deregulation in multiple cellular signaling pathways. It was of no surprise that signaling cascades downstream of growth factor receptors were inhibited by DIM. Down-regulation of EGFR mutant activity and protein levels by DIM resulted in diminished Akt activity, enhanced expression levels of p21 or p27, and reduction of CDK4 and CDK6, which subsequently attenuates the progression from the G1 to S phase of the cell cycle. DIM treatment also induced PARP1 cleavage, reduced the expression of Surviving, and modulated the apoptotic pathway to achieve the inhibition of cancer cell proliferation.

Suppression of the proliferative p44/42 MAPK pathway by DIM treatment was also observed in all the tested cell lines, with the exception of the H4 glioma cells with and without EGFRvIII expression, which had an increased activation of p44/42 MAPK activity. This increased p44/42 MAPK activity in H4 glioma cells resulted in an up-regulation of Cyclin D1. Although this result is counter-intuitive, it has been shown that the blockade of the Akt pathway with pharmacologic agents often results in increased activation of the p44/42 MAPK pathway [51]. This feed-back mechanism may be a characteristic of cancer cells which have the ability to activate different signaling pathways in order to have a survival or growth advantage under stress conditions. Nevertheless, DIM-mediated down-regulation of the Akt pathway was able to alter the expression of cell cycle regulators and induce apoptotic changes to overcome this feedback regulation. Furthermore, early studies showed that DIM treatment induces activation of the p38 MAPK pathway, leading to apoptosis of cancer cells [29;41;42]. This stress activated pathway may play a significant role in DIM-mediated inhibition of ErbB-receptor expression as activation of this pathway has been shown to induce internalization and degradation of EGFR [52;53]. Activation of p38 MAPK pathway may also play a role in DIM-mediated down-regulation of EGFR mutants as activation of p38 MAPK upon DIM treatments was clearly evident in these drug-resistant EGFR mutant-expressing cancer cell lines.

Taken together, we demonstrated that DIM can down-regulate multiple oncogenes and block multiple important signaling pathways that are responsible for promoting cancer cell survival and growth. Most importantly, our findings suggest significant potential clinical benefits as previous results have shown that EGFRvIII expression in cancer cells not only increases the invasiveness of cancer cells, it also renders these cells resistant to conventional cancer treatments such as chemotherapy and radiation. Non-small cell lung cancer cells also expressing EGFR mutations (T790M) are also resistant to TKIs, but DIM may be used as an alternative to inhibit the growth of these tumors.

An additional clinical benefit may be the prevention of metastatic disease or recurrence as DIM has the ability to decrease the invasive potential of aggressive cancer cells expressing EGFR mutants. The mechanism by which DIM inhibits cancer cell migration/invasion is largely unknown, although a few reports suggest the DIM suppresses the expression of the pro-metastatic chemokine receptor CXCR4 and its ligand CXCL12 [54]. Therefore, non-toxic natural products from natural resources could be useful in combination with conventional chemotherapeutic agents for the treatment of drug-resistant human cancers with lower toxicity and higher efficacy. Our future studies will address the mechanism by which DIM inhibits the enhanced invasive potential mediated by EGFRvIII in cancer cells and whether DIM alone or in combination with other targeted therapies can inhibit increased tumorigenesis associated with cancer cells expressing EGFR mutants.

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References

- Schmidt M, Lichtner RB. EGF receptor targeting in therapy-resistant human tumors. *Drug Resist Updat* 2002;5:11–18. [PubMed: 12127860]
- Chan SK, Hill ME, Gullick WJ. The role of the epidermal growth factor receptor in breast cancer. *J Mammary Gland Biol Neoplasia* 2006;11:3–11. [PubMed: 16947082]
- Gee JM, Robertson JF, Gutteridge E, Ellis IO, Pinder SE, Rubini M, Nicholson RI. Epidermal growth factor receptor/HER2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. *Endocr Relat Cancer* 2005;12 1:S99–S111. [PubMed: 16113104]
- Chakravarti A, Dicker A, Mehta M. The contribution of epidermal growth factor receptor (EGFR) signaling pathway to radioresistance in human gliomas: a review of preclinical and correlative clinical data. *Int J Radiat Oncol Biol Phys* 2004;58:927–931. [PubMed: 14967452]
- Huang PH, Cavenee WK, Furnari FB, White FM. Uncovering therapeutic targets for glioblastoma: a systems biology approach. *Cell Cycle* 2007;6:2750–2754. [PubMed: 17986864]
- Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J, Haber DA. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139. [PubMed: 15118073]
- Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE, Meyerson M. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–1500. [PubMed: 15118125]
- Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M, Varmus H. EGF receptor gene mutations are common in lung cancers from “never smokers” and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 2004;101:13306–13311. [PubMed: 15329413]
- Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 2004;305:1163–1167. [PubMed: 15284455]
- Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. *Pharmacol Res* 2007;55:224–236. [PubMed: 17317210]
- Safe S, Papineni S, Chintharlapalli S. Cancer chemotherapy with indole-3-carbinol, bis(3'-indolyl)methane and synthetic analogs. *Cancer Lett* 2008;269:326–338. [PubMed: 18501502]
- Chen I, McDougal A, Wang F, Safe S. Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. *Carcinogenesis* 1998;19:1631–1639. [PubMed: 9771935]

13. Ichite N, Chougule MB, Jackson T, Fulzele SV, Safe S, Singh M. Enhancement of docetaxel anticancer activity by a novel diindolymethane compound in human non-small cell lung cancer. *Clin Cancer Res* 2009;15:543–552. [PubMed: 19147759]
14. Kassie F, Anderson LB, Scherber R, Yu N, Lahti D, Upadhyaya P, Hecht SS. Indole-3-carbinol inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone plus benzo(a)pyrene-induced lung tumorigenesis in A/J mice and modulates carcinogen-induced alterations in protein levels. *Cancer Res* 2007;67:6502–6511. [PubMed: 17616712]
15. McDougal A, Gupta MS, Morrow D, Ramamoorthy K, Lee JE, Safe SH. Methyl-substituted diindolymethanes as inhibitors of estrogen-induced growth of T47D cells and mammary tumors in rats. *Breast Cancer Res Treat* 2001;66:147–157. [PubMed: 11437101]
16. Qin C, Morrow D, Stewart J, Spencer K, Porter W, Smith R III, Phillips T, Abdelrahim M, Samudio I, Safe S. A new class of peroxisome proliferator-activated receptor gamma (PPARgamma) agonists that inhibit growth of breast cancer cells: 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes. *Mol Cancer Ther* 2004;3:247–260. [PubMed: 15026545]
17. Chang X, Tou JC, Hong C, Kim HA, Riby JE, Firestone GL, Bjeldanes LF. 3,3'-Diindolymethane inhibits angiogenesis and the growth of transplantable human breast carcinoma in athymic mice. *Carcinogenesis* 2005;26:771–778. [PubMed: 15661811]
18. McGuire KP, Ngoubilly N, Neavyn M, Lanza-Jacoby S. 3,3'-diindolymethane and paclitaxel act synergistically to promote apoptosis in HER2/Neu human breast cancer cells. *J Surg Res* 2006;132:208–213. [PubMed: 16580691]
19. Rahman KM, Ali S, Aboukameel A, Sarkar SH, Wang Z, Philip PA, Sakr WA, Raz A. Inactivation of NF-kappaB by 3,3'-diindolymethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells. *Mol Cancer Ther* 2007;6:2757–2765. [PubMed: 17913854]
20. Firestone GL, Bjeldanes LF. Indole-3-carbinol and 3-3'-diindolymethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. *J Nutr* 2003;133:2448S–2455S. [PubMed: 12840223]
21. Hong C, Kim HA, Firestone GL, Bjeldanes LF. 3,3'-Diindolymethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21 (WAF1/CIP1) expression. *Carcinogenesis* 2002;23:1297–1305. [PubMed: 12151347]
22. Rahman KW, Li Y, Wang Z, Sarkar SH, Sarkar FH. Gene expression profiling revealed survivin as a target of 3,3'-diindolymethane-induced cell growth inhibition and apoptosis in breast cancer cells. *Cancer Res* 2006;66:4952–4960. [PubMed: 16651453]
23. Vanderlaag K, Samudio I, Burghardt R, Barhoumi R, Safe S. Inhibition of breast cancer cell growth and induction of cell death by 1,1-bis(3'-indolyl)methane (DIM) and 5,5'-dibromoDIM. *Cancer Lett* 2006;236:198–212. [PubMed: 16051428]
24. Vanderlaag K, Su Y, Frankel AE, Grage H, Smith R III, Khan S, Safe S. 1,1-Bis(3'-indolyl)-1-(p-substituted phenyl)methanes inhibit proliferation of estrogen receptor-negative breast cancer cells by activation of multiple pathways. *Breast Cancer Res Treat* 2008;109:273–283. [PubMed: 17624585]
25. Wang Z, Yu BW, Rahman KM, Ahmad F, Sarkar FH. Induction of growth arrest and apoptosis in human breast cancer cells by 3,3-diindolymethane is associated with induction and nuclear localization of p27kip. *Mol Cancer Ther* 2008;7:341–349. [PubMed: 18281517]
26. Hong C, Firestone GL, Bjeldanes LF. Bcl-2 family-mediated apoptotic effects of 3,3'-diindolymethane (DIM) in human breast cancer cells. *Biochem Pharmacol* 2002;63:1085–1097. [PubMed: 11931841]
27. Riby JE, Xue L, Chatterji U, Bjeldanes EL, Firestone GL, Bjeldanes LF. Activation and potentiation of interferon-gamma signaling by 3,3'-diindolymethane in MCF-7 breast cancer cells. *Mol Pharmacol* 2006;69:430–439. [PubMed: 16267208]
28. Wang TT, Milner MJ, Milner JA, Kim YS. Estrogen receptor alpha as a target for indole-3-carbinol. *J Nutr Biochem* 2006;17:659–664. [PubMed: 16488130]
29. Xue L, Firestone GL, Bjeldanes LF. DIM stimulates IFNgamma gene expression in human breast cancer cells via the specific activation of JNK and p38 pathways. *Oncogene* 2005;24:2343–2353. [PubMed: 15735741]

30. Rahman KW, Sarkar FH. Inhibition of nuclear translocation of nuclear factor- κ B contributes to 3,3'-diindolylmethane-induced apoptosis in breast cancer cells. *Cancer Res* 2005;65:364–371. [PubMed: 15665315]
31. Ali S, Banerjee S, Ahmad A, El-Rayes BF, Philip PA, Sarkar FH. Apoptosis-inducing effect of erlotinib is potentiated by 3,3'-diindolylmethane in vitro and in vivo using an orthotopic model of pancreatic cancer. *Mol Cancer Ther* 2008;7:1708–1719. [PubMed: 18566242]
32. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by AKT and NF- κ B pathways in prostate cancer cells. *Front Biosci* 2005;10:236–243. [PubMed: 15574364]
33. Moiseeva EP, Heukers R, Manson MM. EGFR and Src are involved in indole-3-carbinol-induced death and cell cycle arrest of human breast cancer cells. *Carcinogenesis* 2007;28:435–445. [PubMed: 16956907]
34. Tang CK, Gong XQ, Moscatello DK, Wong AJ, Lippman ME. Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer. *Cancer Res* 2000;60:3081–3087. [PubMed: 10850460]
35. Zhang Y, Su H, Rahimi M, Tochiara R, Tang C. EGFRvIII-induced estrogen-independence, tamoxifen-resistance phenotype correlates with PgR expression and modulation of apoptotic molecules in breast cancer. *Int J Cancer* 2009;125:2021–2028. [PubMed: 19588487]
36. Eder JP, Vande Woude GF, Boerner SA, LoRusso PM. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. *Clin Cancer Res* 2009;15:2207–2214. [PubMed: 19318488]
37. Kong DS, Song SY, Kim DH, Joo KM, Yoo JS, Koh JS, Dong SM, Suh YL, Lee JI, Park K, Kim JH, Nam DH. Prognostic significance of c-Met expression in glioblastomas. *Cancer* 2009;115:140–148. [PubMed: 18973197]
38. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist* 2009;14:320–368. [PubMed: 19346299]
39. Chen HJ, Mok TS, Chen ZH, Guo AL, Zhang XC, Su J, Wu YL. Clinicopathologic and Molecular Features of Epidermal Growth Factor Receptor T790M Mutation and c-MET Amplification in Tyrosine Kinase Inhibitor-resistant Chinese Non-small Cell Lung Cancer. *Pathol Oncol Res*. 2009
40. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Janne PA. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–1043. [PubMed: 17463250]
41. Khwaja FS, Wynne S, Posey I, Djakiew D. 3,3'-diindolylmethane induction of p75NTR-dependent cell death via the p38 mitogen-activated protein kinase pathway in prostate cancer cells. *Cancer Prev Res (Phila Pa)* 2009;2:566–571. [PubMed: 19470787]
42. Vivar OI, Lin CL, Firestone GL, Bjeldanes LF. 3,3'-Diindolylmethane induces a G(1) arrest in human prostate cancer cells irrespective of androgen receptor and p53 status. *Biochem Pharmacol* 2009;78:469–476. [PubMed: 19433067]
43. Cai XM, Tao BB, Wang LY, Liang YL, Jin JW, Yang Y, Hu YL, Zha XL. Protein phosphatase activity of PTEN inhibited the invasion of glioma cells with epidermal growth factor receptor mutation type III expression. *Int J Cancer* 2005;117:905–912. [PubMed: 15986432]
44. Choe G, Park JK, Jouben-Steele L, Kremen TJ, Liao LM, Vinters HV, Cloughesy TF, Mischel PS. Active matrix metalloproteinase 9 expression is associated with primary glioblastoma subtype. *Clin Cancer Res* 2002;8:2894–2901. [PubMed: 12231534]
45. Damstrup L, Wandahl PM, Bastholm L, Elling F, Skovgaard PH. Epidermal growth factor receptor mutation type III transfected into a small cell lung cancer cell line is predominantly localized at the cell surface and enhances the malignant phenotype. *Int J Cancer* 2002;97:7–14. [PubMed: 11774237]
46. Lal A, Glazer CA, Martinson HM, Friedman HS, Archer GE, Sampson JH, Riggins GJ. Mutant epidermal growth factor receptor up-regulates molecular effectors of tumor invasion. *Cancer Res* 2002;62:3335–3339. [PubMed: 12067969]
47. Yu H, Gong X, Luo X, Han W, Hong G, Singh B, Tang CK. Co-expression of EGFRvIII with ErbB-2 enhances tumorigenesis: EGFRvIII mediated constitutively activated and sustained signaling

- pathways, whereas EGF-induced a transient effect on EGFR-mediated signaling pathways. *Cancer Biol Ther* 2008;7
48. Han W, Zhang T, Yu H, Foulke JG, Tang CK. Hypophosphorylation of residue Y1045 leads to defective downregulation of EGFRvIII. *Cancer Biol Ther* 2006;5:1361–1368. [PubMed: 16969069]
49. Karamouzis MV, Konstantinopoulos PA, Papavassiliou AG. Targeting MET as a strategy to overcome crosstalk-related resistance to EGFR inhibitors. *Lancet Oncol* 2009;10:709–717. [PubMed: 19573800]
50. Lal B, Goodwin CR, Sang Y, Foss CA, Cornet K, Muzamil S, Pomper MG, Kim J, Laterra J. EGFRvIII and c-Met pathway inhibitors synergize against PTEN-null/EGFRvIII+ glioblastoma xenografts. *Mol Cancer Ther* 2009;8:1751–1760. [PubMed: 19584231]
51. Kinkade CW, Castillo-Martin M, Puzio-Kuter A, Yan J, Foster TH, Gao H, Sun Y, Ouyang X, Gerald WL, Cordon-Cardo C, bate-Shen C. Targeting AKT/mTOR and ERK MAPK signaling inhibits hormone-refractory prostate cancer in a preclinical mouse model. *J Clin Invest* 2008;118:3051–3064. [PubMed: 18725989]
52. Frey MR, Dise RS, Edelblum KL, Polk DB. p38 kinase regulates epidermal growth factor receptor downregulation and cellular migration. *EMBO J* 2006;25:5683–5692. [PubMed: 17139251]
53. Vergarajauregui S, San MA, Puertollano R. Activation of p38 mitogen-activated protein kinase promotes epidermal growth factor receptor internalization. *Traffic* 2006;7:686–698. [PubMed: 16683917]
54. Hsu EL, Chen N, Westbrook A, Wang F, Zhang R, Taylor RT, Hankinson O. CXCR4 and CXCL12 down-regulation: a novel mechanism for the chemoprotection of 3,3'-diindolylmethane for breast and ovarian cancers. *Cancer Lett* 2008;265:113–123. [PubMed: 18378071]

Fig. 1.

The effect of DIM on the proliferation of drug-resistant cancer cells expressing EGFR mutants. The growth of breast cancer (MDA-MB-361 and MCF-7) (A), glioma (H4 and U87MG) (B), and non-small cell lung cancer [H1650 (delE746-A750) and H1975 (L858R+T790M)] (C) cells was inhibited by DIM. EGFRvIII-expressing breast cancer cells (A) and H1975 lung cancer cells (C) were slightly less sensitive to growth inhibition by DIM, while EGFRvIII-expressing glioma cells (B) were as sensitive as parental cells. Low ErbB2-expressing MCF-7 breast cancer cells were also less sensitive to growth inhibition by DIM (A). Cells were plated and 24 hours later were treated with 10 and 20 μM DIM (breast), 20 and 30 μM DIM (glioma), or 20 and 50 μM DIM (lung) for 1, 2, and 3 days. Vehicle (DMSO) treated cells were used as controls. Cells were counted by a coulter counter. “361” represents MDA-MD-361 breast cancer cells; “U87” represents U87MG glioma cells; “wt” represents parental cells; “V” represents empty-vector-expressing cells; and “vIII” represents EGFRvIII-expressing cells.

Fig. 2.

The effect of DIM on receptor tyrosine kinases in drug-resistant cancer cells expressing EGFR mutants. Immunoblot analysis of lysates from breast cancer (A), glioma (B), and non-small cell lung cancer (C) cells treated with 20 μ M (breast), 30 μ M (glioma), or 50 μ M (lung) DIM for 48 hours showing that the activation and/or expression of EGFR, EGFRvIII, ErbB2, and Met is decreased by DIM.

Fig. 3.

The effect of DIM on the activation of survival/proliferative/stress signaling pathways in drug-resistant cancer cells expressing EGFR mutants. Immunoblot analysis of lysates from breast cancer (A), glioma (B), and non-small cell lung cancer (C) cells treated with 20 μ M (breast), 30 μ M (glioma), or 50 μ M (lung) DIM for 48 hours showing that the activation and/or expression of Akt and p44/42 MAPK is decreased by DIM in cancer cells expressing EGFR mutants. DIM also activates the stress associated p38 MAPK pathway in these cells.

Fig. 4.

The effect of DIM on cell cycle regulators in drug-resistant cancer cells expressing EGFR mutants. Immunoblot analysis of lysates from breast cancer (A), glioma (B), and non-small cell lung cancer (C) cells treated with 20 μ M (breast), 30 μ M (glioma), or 50 μ M (lung) DIM for 48 hours showing that the DIM induces the expression of cell cycle inhibitors (p27 and p21), while decreasing the expression of Cyclin D1 and cyclin-dependent kinases (CDK6 and CDK4) in cancer cells expressing EGFR mutants.

Fig. 5.

The effect of DIM on apoptotic molecules in drug-resistant cancer cells expressing EGFR mutants. Immunoblot analysis of lysates from breast cancer (A), glioma (B), and non-small cell lung cancer (C) cells treated with 20 μ M (breast), 30 μ M (glioma), or 50 μ M (lung) DIM for 48 hours showing that the DIM induces the cleavage of PARP1 and a reduction in PARP2 expression. DIM also decreases the expression of the anti-apoptotic Survivin and alters the balance of pro- (Bad and Bax) and anti- (Bcl-2 and Bcl-xL) apoptotic proteins- favoring apoptosis.

Fig. 6.

The effect of DIM on the invasive potential of drug-resistant cancer cells expressing EGFR mutants. EGFRvIII-expressing MDA-MB-361 breast cancer (A) and H4 glioma (B) cells as well as H1650 and H1975 lung cancer cells (C) have significantly reduced invasion after being treated with DIM (* = $p < 0.01$; ** = $p < 0.001$; ANOVA). DIM also significantly reduced the invasion of H4 parental cells (B). Cells were plated in 0.1% BSA/IMEM in the upper chamber of transwells with membranes with 8 μ m pores, and with serum containing media in the lower chamber. DIM was used as pre-treatments and added to the upper chamber of the transwells containing the cells. After 48 hours of incubation, the membranes were fixed and stained, and cells were counted on the underside of the membranes. The results shown are the mean and SE from 10 fields counted in triplicate filters.

Table 1

Drug-resistant phenotypes of EGFR mutant-expressing cancer cells

Malignancy	Cell Line	EGFR Mutant	Phenotype	Reference(s)
Breast Cancer	MDA-MB-361	EGFRvIII	Tamoxifen-Resistant	Zhang et al. 2009
Glioblastoma	U87MG	EGFRvIII	Radiation/Chemotherapy-Resistant	Schmidt et al. 2002 Chakravarti et al. 2004 Huang et al. 2007
Non-Small Cell Lung Cancer	H1650	delE746-A750	Tyrosine Kinase Inhibitor-Sensitive	Lynch et al. 2004 Paez et al. 2004 Pao et al. 2004 Sordella et al. 2004
	H1975	L858R+Y790M	Tyrosine Kinase Inhibitor-Resistant	