

3, 3'-diindolylmethane Enhances the Effectiveness of Herceptin against HER-2/Neu-Expressing Breast Cancer Cells

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Abstract

Herceptin failure is a major clinical problem in breast cancer. A subset of breast cancer patients with high *HER-2/neu* levels eventually experience metastatic disease progression when treated with Herceptin as a single agent. Mechanistic details of development of this aggressive disease are not clear. Therefore, there is a dire need to better understand the mechanisms by which drug resistance develops and to design new combined treatments that benefit patients with aggressive breast cancer and have minimal toxicity. We hypothesized that 3, 3'-diindolylmethane (DIM), a non-toxic agent can be combined with Herceptin to treat breast cancers with high levels of *HER-2/neu*. Here, we evaluated the effects of Herceptin alone and in combination with DIM on cell viability, apoptosis and clonogenic assays in SKBR3 (*HER-2/neu*-expressing) and MDA-MB-468 (*HER-2/neu* negative) breast cancer cells. We found that DIM could enhance the effectiveness of Herceptin by significantly reducing cell viability, which was associated with apoptosis-induction and significant inhibition of colony formation, compared with single agent treatment. These results were consistent with the down-regulation of *Akt* and *NF-κB p65*. Mechanistic investigations revealed a significant upregulation of *miR-200* and reduction of *FoxM1* expression in DIM and Herceptin-treated breast cancer cells. We, therefore, transfected cells with pre-miR-200 or silenced *FoxM1* in these cells for understanding the molecular mechanism involved. These results provide experimental evidence, for the first time, that DIM plus Herceptin therapy could be translated to the clinic as a therapeutic modality to improve treatment outcome of patients with breast cancer, particularly for the patients whose tumors express high levels of *HER-2/neu*.

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Introduction

Herceptin (Trastuzumab), a humanized monoclonal antibody targeting *HER-2/neu*, has shown some benefit as a treatment for patients with *HER-2/neu*-expressing breast cancer, but clinical studies show that patients with high levels of *HER-2/neu* who are treated with a single dose of Herceptin progress to metastatic disease within one year [1–5]. The potential mechanisms underlying Herceptin failure are found in altered *EGFR* receptors, increased *Akt* activity and *IGF-1R* signaling, reduced *p27^{kip1}* and *PTEEN* level in breast cancer cell [3,6]. Interestingly, these signaling pathways have been reported to be modulated by a natural non-toxic agent, 3, 3'-diindolylmethane (DIM) [7–9] which raises the possibility that combination of DIM with Herceptin might help to enhance the antitumor activity of Herceptin against *HER-2/neu*-expressing breast cancer. Nevertheless, as not much is known about the mechanism(s) that lead to the development of aggressive disease, the mechanisms by which DIM and Herceptin may affect this process is unclear. Our current study provides new insight into the mechanism(s) of this drug combination and, therefore, has the

potential of advancing our understanding of the biology of aggressive breast cancer.

Dietary and epidemiological studies suggest that consumption of vegetables and fruits are associated with reduced risks for certain cancers, including breast cancer [10–12]. DIM is a plant-derived, natural autolytic compound found in cruciferous vegetables that selectively kills breast cancer cells without affecting normal cells [13–18] and enhances the efficacy of chemotherapeutic agents [13,14,19]. Previously we showed that DIM could reduce the concentration of Taxotere needed to induce an inhibitory effect on breast cancer cells [13,14,17,19], which raises the possibility that combination of DIM with Herceptin might help overcome the shortcomings (both toxicity and molecular cause of Herceptin failure) and increased higher efficacy of Herceptin. We believe that combination of DIM and Herceptin is more potent than individual compounds, which could maximize the effect of Herceptin-based therapies and target multiple signaling pathways.

MicroRNAs (miRNAs) are small non-coding RNAs that play an important role in modulating multiple cellular pathways, including cell proliferation, differentiation, and apoptosis, and thus may

function as oncogenes or tumor suppressor genes [20]. Among them, changes in miR-200 (*miR-200a*, *miR-200b* and *miR-200c*) levels have been associated with enhanced tumorigenesis and significantly correlated with decreased survival [21–24]. Furthermore, down-regulation of *miR-200* is observed in aggressive breast cancer [21,25–27]. *FoxM1*, an oncogenic transcription factor is known to play important role in the development and progression of many malignancies including breast cancer [28–31]. Interestingly, it has been indicated that over-expression of *FoxM1* could lead to decreased expression of miRNAs including *miR-200* [32,33]. With an inverse relationship between *FoxM1* and *miR-200*, the increased expression of *FoxM1*, promotes oncogenesis and progression of various carcinomas, and contributes to chemotherapeutic resistance. However, the interrelationship between *FoxM1* and *miR-200* that are involved in progression of breast cancer has not yet been clarified. Furthermore, *FoxM1* has been shown to confer resistance to Herceptin and microtubule-stabilizing drug Paclitaxel in breast cancer cells [34]. Our recent studies have shown that inactivation of *FoxM1*, and its target genes, by DIM could enhance the therapeutic efficacy of Taxotere in breast and prostate cancer cells [19] suggesting that DIM in combination with Herceptin could be useful to upregulate *miR-200* and down-regulate *FoxM1* which should help to develop therapeutic strategies for the prevention and/or treatment of breast cancer.

Here, we report for the first time that DIM upregulates *miRNA-200* and down-regulates *FoxM1* in *HER-2/neu* expressing, SKBR3 breast cancer cells. We also report that DIM has moderated effect on *miR-200* and *FoxM1* in *HER-2/neu* negative, MDA-MB-468 breast cancer cells. More importantly, combination of DIM and Herceptin is much more effective than either agent alone in *HER-2/neu* expressing, breast cancer cells, suggesting that combination-mediated alterations in *miR-200*, along with inactivation of *Akt* and *NF- κ B p65*, could be a novel approach for the treatment of patients with breast cancer, particularly for the patients whose tumors express high levels of *HER-2/neu*.

Materials and Methods

Cell Lines and Reagents

Breast cancer cell lines, SKBR3 (*HER-2/neu*-expressing) and MDA-MB-468 (*HER-2/neu* negative) were obtained from ATCC (Manassas, VA). The cell lines have been tested and authenticated in core facility Applied Genomics Technology Center at Wayne State University. Primary antibodies for *FoxM1*, *Akt*, *pAkt*, *NF- κ B p65* and anti-poly (ADP-ribose) polymerase (*PARP*) were purchased from Santa Cruz Biotechnology and anti- β -actin was purchased from Sigma-Aldrich (St. Louis, MO). All secondary antibodies were obtained from Pierce. *FoxM1* siRNA and control siRNA were obtained from Santa Cruz Biotechnology. LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA). Chemiluminescence detection of proteins was done with a kit from Amersham Biosciences (Piscataway, NJ). Protease inhibitor cocktail, MTT reagent and all other chemicals were obtained from Sigma (St. Louis, MO). DIM (marketed as BR-DIM with enhanced bioavailability), generously provided by Dr. Michael Zeligs (BioResponse, CO), was dissolved in DMSO to make a 50 mmol/L stock solution. Herceptin (Genentech, Inc) was provided by Karman Cancer Institute dissolved in Bacteriologic water and BFWI (1.1% benzyl alcohol) to make 21 mg/ml stock solution.

Cell Viability Assay

Cells were seeded in 96-well plates. After 24 hours, they were treated with DIM (10, 15 or 20 μ M) followed by treatment with

Herceptin (0.25, 0.75 or 1.00 μ g/ml) for 24, 48 or 72 hours. Cell growth studies were performed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) as described earlier [13,15].

Clonogenic Assay

Survival of breast cells was tested by clonogenic assay, as described before [17,21]. Briefly, cells were plated in 6-well plates, treated, trypsinized, re-plated in 100-mm Petri dishes and cultured at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator. Colonies were stained with 2% crystal violet, counted, and quantitated.

Quantification of Apoptosis by Enzyme-linked Immunosorbent Assay

Cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science) was used to detect apoptosis. Cells, seeded in six-well plates, were treated with 15 μ M DIM, 0.75 μ g/ml Herceptin or their combination for 48 h, trypsinized, and ~10,000 cells were assayed as described earlier [13,14,16]. TECAN's microplate fluorometer (TECAN) was used to measure color intensity at 405 nm.

Western Blot Analysis

Cells were lysed in 62.5 mmol/L Tris-HCl and 2% SDS, and protein concentration was measured using BCA protein assay (Pierce). Proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. Membranes were incubated with specific primary antibodies, secondary antibody conjugated with peroxidase and the signal was detected using the chemiluminescent detection system (Pierce) as described earlier [16,19,35].

RNA Isolation and Real-time Reverse Transcription-PCR (RT-PCR) Analysis

Total RNA from DIM and Herceptin-treated cells was isolated by TRIzol (Invitrogen) according to the manufacturer's protocol and subjected to analyses by RT-PCR, as described earlier [36].

miRNA Real-time Reverse Transcription-PCR

To verify the alterations in the expression of *miR-200* in DIM and Herceptin-treated breast cancer cells, we performed RT-PCR analysis using TaqMan MicroRNA Assay Kit (Applied Biosystems) following the manufacturer's protocol. RT reaction conditions and primers used in this study were, exactly the same as described previously [21]. Briefly, 5 ng of total RNA from each sample were subjected to reverse transcription with a specific miRNA primer (Applied Biosystems). Real-time PCRs were then carried out in a total of 25 μ L reaction mixture in SmartCycler II (Cepheid). Data were analyzed according to the comparative Ct method and normalized by RNU48 expression in each sample.

Transfection with Pre-/anti-miRNAs

We transfected SKBR3 and MDA-MB-468 cells with a cocktail of pre-miRNAs (*pre-miR-200a+pre-miR-200b+pre-miR-200c*) or anti-miRNAs (*anti-miR-200a+anti-miR-200b+anti-miR-200c*) (Applied Biosystems), as indicated for individual experiments [21]. Briefly, cells were seeded at 5×10^5 cells per well in six-well plates and transfected with pre-miR-200s/anti-miR-200s or non-specific pre-/anti-miRNAs control (Ambion, Austin, TX) at a final concentration of 20 nM using DharmaFECT2 transfection reagent (Dharmacon) for 72 h before analyses. At the end of transfection, cells were trypsinized, re-seeded in six well plates and re-transfected for a total of three rounds of transfections. 72 hours

after the last transfection, cells were seeded in 96-well plates for MTT assay and multiple wells of six well plates for real time RT-PCR and western blot analysis.

FoxM1 siRNA Transfection

To determine the effect of FoxM1 siRNA transfection, cells were plated in 100 mm petri dishes overnight and transfected with 100 nmol/l of FoxM1 siRNA or the control siRNA for 48 h by Lipofectamine 2000 following the manufacturer's protocol.

Statistical Analysis

The data are presented as the mean values \pm SE. Comparisons between groups were evaluated by Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

Results

Cell Growth Inhibition by DIM and Herceptin Treatment

We tested several doses of DIM and Herceptin at different time points (data not shown) and found that treatment of SKBR3 and MDA-MB-468 breast cancer cells with 15 μ M DIM for 72 h caused 40–60% growth inhibition which is irrespective of *HER-2/neu* status. However, a combination of these doses resulted in 80% growth inhibition of SKBR3 breast cancer cells (Fig. 1A), suggesting a significant inhibitory effect of combination treatment in *HER-2/neu*-expressing breast cancer cells. As observed in the MTT assays, treatment with Herceptin alone did not show any decreased cell viability in MDA-MB-468 (*HER-2/neu* negative) breast cancer cells which confirms the specificity of Herceptin action. Also, DIM and Herceptin combination did not show any significant decrease of cell viability in MDA-MB-468 breast cancer cells, compared to DIM alone. Previous studies have shown the effects of different doses of Herceptin in breast cancer cells [37]. Our results show that the combination of DIM with a lower dose of Herceptin elicited significantly greater inhibition of cancer cell growth compared with either agent alone (Fig. 1A).

Effect of DIM and Herceptin on Clonogenicity of Breast Cancer Cells

The effect of DIM and Herceptin treatment on anchorage-dependent clonogenic growth was assessed by clonogenic assay (Fig. 1B). Treatment with 15 μ M DIM and 0.75 μ g/ml Herceptin resulted in a significant inhibition (90%) of colony formation in SKBR3 cells when compared with single-agent treatment (Fig. 1C). On the other hand there was no significant decrease in number of colonies in MDA-MB-468 breast cancer cells (Fig. 1C).

Induction of Apoptosis by DIM and Herceptin

The inhibition of cell viability was further evaluated by determining the apoptotic effects, using the cell death detection ELISA. Here we found that the combination of DIM (15 μ M) and Herceptin (0.75 μ g/ml) resulted in a significant induction of apoptosis in SKBR3 breast cancer cell lines tested (DIM, 2.25-fold; Herceptin, 4.0-fold; and DIM plus Herceptin, 8.25-fold) (Fig. 2A). In contrast, we found apoptotic effects of DIM in MDA-MB-468 cells. However, combination treatment did not induce apoptosis in MDA-MB-468 cells. Fold changes in DIM and Herceptin-treated SKBR3 and MDA-MB-468 cells relative to untreated cells are shown in (Fig. 2A). We also observed that the DIM and Herceptin combination treatment resulted in significantly more cleaved PARP compared with mono-treatment (Fig. 2B), suggesting that the combination treatment could induce greater apoptosis in breast cancer cells, particularly in *HER-2/neu* expressed breast

cancer cells. These results are consistent with the cell viability assay. Since inhibition of cell proliferation observed by MTT and clonogenic assay could be due to altered regulation of several genes such as *FoxM1*, *Akt* and *NF- κ B p65*, the underlying mechanism for the inhibition of cell viability was further studied by western blot analysis to determine the effects of DIM and Herceptin on the expression of selective proteins.

Effects of DIM and Herceptin on the Expression of Selective Proteins

Previously we reported that DIM down-regulates *FoxM1* in *HER-2/neu*-expressing breast cancer cells [19]. Thus, we hypothesized that combining DIM with Herceptin may be an effective strategy for treating *HER-2/neu*-expressing breast cancer. *Akt* and *NF- κ B* play a critical role in cancer progression and our previous report suggests that *Akt/NF- κ B* could be efficiently inactivated by DIM [13,15]. We further hypothesized that down-regulation of *FoxM1* signaling by DIM and Herceptin will induce apoptotic cell death, which could be due to inactivation of *FoxM1* directly and indirectly via inactivation of *Akt* and *NF- κ B*. However, a more detailed understanding of the effects of this drug combination and its mechanism of action in *HER-2/neu*-expressing breast cancer is needed. Therefore, to study the functional relevance of DIM and Herceptin-mediated alteration of *FoxM1*, *Akt* and *NF- κ B* expression in breast cancer cells, we treated cells with DIM and Herceptin for 72 hours. Expression of *FoxM1* and *pAkt* proteins was significantly reduced in SKBR3 breast cancer cells treated with DIM and Herceptin when compared with control and single treatment (Fig. 3). Importantly, we found significant down-regulation of FoxM1 in DIM and Herceptin-treated SKBR3 breast cancer cells. Interestingly, we found some inhibitory effect of DIM and Herceptin on *FoxM1* in MDA-MB-468 breast cancer cells. Moreover, we did not find any alterations in the total protein expression of *Akt* in DIM and Herceptin-treated SKBR3 and MDA-MB-468 breast cancer cells (Fig. 3). Since *NF- κ B* was not found to be a significantly-changed protein after DIM and Herceptin treatment in SKBR3 and MDA-MB-468, we did not present this data in Fig. 3. Further, *FoxM1* has been found to be elevated in advanced cancers, including breast carcinoma [8,14,19] and our current results further underline the importance of *FoxM1* in Herceptin efficacy as well. Next, we questioned the mechanism by which elevated *FoxM1* levels might regulate the anti-tumor activity of Herceptin. In particular, we looked at the miRNAs that might be regulated by *FoxM1*. There is evidence in literature to suggest a regulatory connection between *FoxM1* and *miR-200* family of miRNAs [33]. Our earlier publications have independently established a role of *FoxM1* and *miR-200* in the aggressiveness of breast cancer cells [16,17,19,21] but a relationship was never studied. Therefore, we further investigated the effects of DIM and Herceptin on miR-200s.

Upregulation of *miR-200* Expression by DIM and Herceptin

We started with an evaluation of basal levels of miR-200s in the two breast cancer cell lines and found that the levels of miR-200s are endogenously high in MDA-MB-468 cells, compared to the SKBR3 cells (Fig. 4A). This is in line with the known epithelial phenotype of MDA-MB-468 cells. We found a significant upregulation of the expression of *miR-200* family in cells treated with either DIM or in combination with Herceptin (Fig. 4A, B). In SKBR3 cells, DIM as well as Herceptin was found to upregulate miR-200s (Fig. 4B) and the combination was found to be even more effective. While *miR-200a* and *miR-200b* were upregulated by

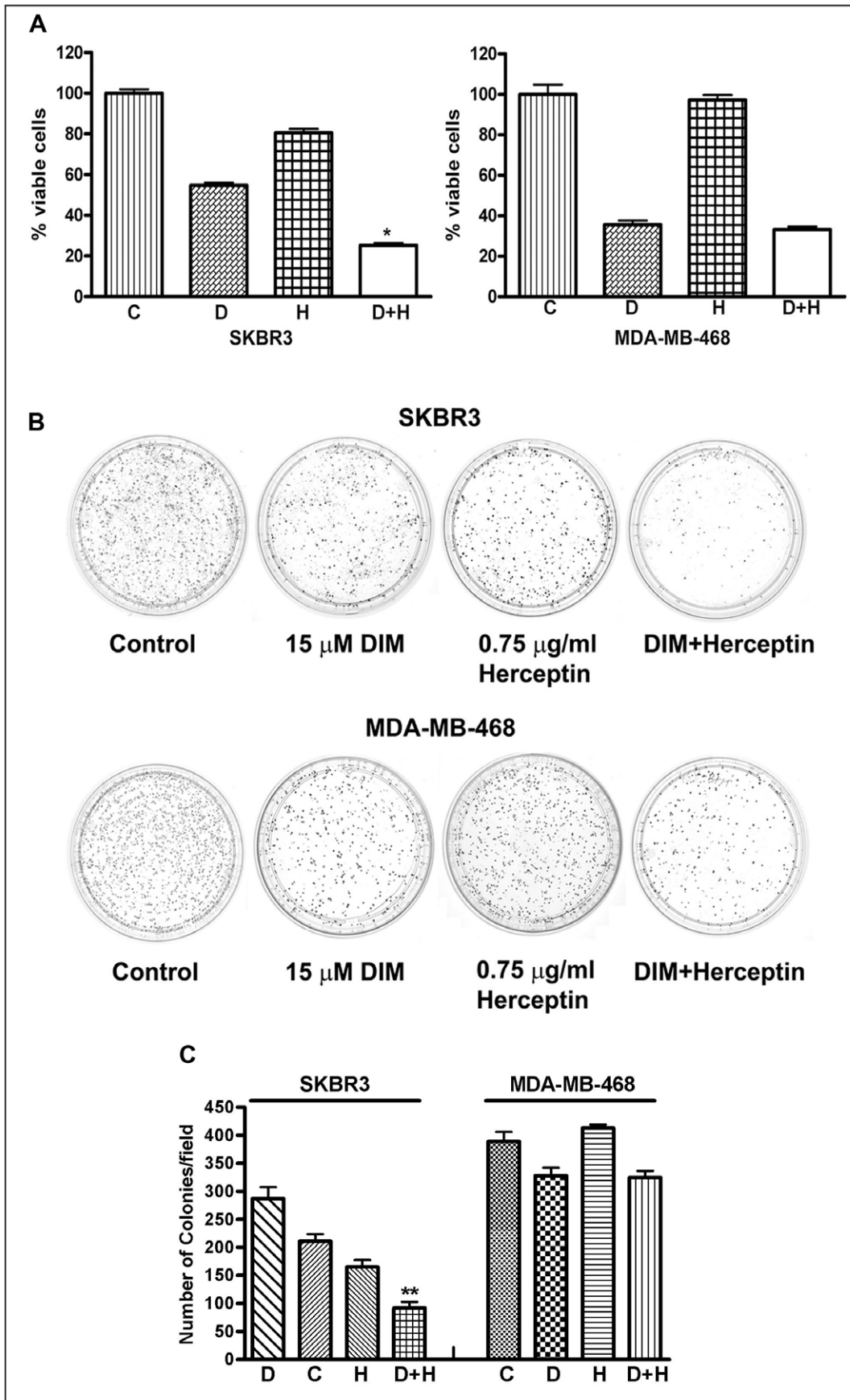


Figure 1. Effects on cell growth after DIM and Herceptin treatment. (A) SKBR3 and MDA-MB-468 cells were treated with DIM (15 μ M) and Herceptin (0.75 μ g/ml), either alone or combination for 72 hours. DIM in combination with Herceptin significantly inhibited cell proliferation, as measured by MTT assay. (B) SKBR3 and MDA-MB-468 cells were treated with 15 μ M DIM, 0.75 μ g/ml Herceptin and combination. Assay for anchorage-dependent clonogenicity was done as described under materials and methods. (C) The bar graphs at the bottom represent quantification of results presented on the top in each case. *, $P < 0.05$; **, $P < 0.01$ relative to control. C, Control; D, DIM; H, Herceptin. doi:10.1371/journal.pone.0054657.g001

DIM and Herceptin individually, we did not observe any effect on *miR-200c* of these compounds individually. However, interestingly, a very significant up-regulation of *miR-200c* was observed in the combinational treatment (Fig. 4B). In MDA-MB-468 cells, DIM alone was found to upregulate *miR-200a*, *miR-200b* but Herceptin alone was found to have no effect and no significant up-regulation of *miR-200c* was observed in the combinational treatment in MDA-MB-468 cells (Fig. 4C). As expected, we did not observe any effect of Herceptin alone on *miR-200a/b* expression in MDA-MB-468 cells. However, surprisingly, in combination with DIM, Herceptin significantly induced the expression levels of *miR-200a* and *miR-200b*. To evaluate an involvement of miR-200s in the cytotoxic action of DIM/Herceptin, we exposed control (transfected with non-specific anti-miRNAs) and anti-miR-200s-transfected SKBR3 cells (transfected with a cocktail containing anti-miR-200a+anti-miR-200b+anti-miR-200c) to DIM plus Herceptin. The cells transfected with non-specific anti-miRNAs were found to be sensitive to DIM plus Herceptin treatment, however, antagonizing miR-200s significantly ($p < 0.01$) attenuated the effects of DIM/Herceptin (Fig. 4D). These results clearly establish

a mechanistic involvement of miR-200s in DIM plus Herceptin. DIM plus Herceptin work via up-regulation of miR-200s, and antagonizing miR-200s (miR-200a+miR-200b+miR-200c) significantly diminishes the biological effects of these drugs. To determine that anti-miR-200s transfections reduced the levels of miR-200 family miRNAs, we evaluated the levels of miR-200a, miR-200b as well as miR-200c in SKBR3 cells transfected with anti-miR-200s (anti-miR-200a+anti-miR-200b+anti-miR-200c) vs. SKBR3 cells transfected with non-specific anti-miRNAs, and found that the transfections with anti-miR-200s lowered the levels of all miR-200s by at least half (Fig. 4E, controls in non-specific anti-miRNAs vs. controls in anti-miR-200s). We also looked at the levels of miR-200s after these cells were exposed to DIM plus Herceptin for 72 hours. The results suggested that while DIM plus Herceptin induced the levels of all miR-200s in control SKBR3 cells (transfected with non-specific anti-miRNAs), the induction in anti-miR-200s-transfected SKBR3 cells was considerably reduced (Fig. 4E). Western blot analysis of these samples indicated that DIM plus Herceptin down-regulated *FoxM1* and *pAkt* levels in SKBR3 cells as expected, and this was attenuated by transfections with anti-miR-200s (Fig. 4F). The reduced induction of miR-200s (miR-200a+miR-200b+miR-200c) and inhibition of *FoxM1/Akt* activation may help explain the attenuation of DIM plus Herceptin action as observed in Fig. 4D. To further validate whether *miR-200* indeed could target the expression levels of *FoxM1*, we investigated the effect of transfection of pre-miRNAs and *FoxM1* siRNA in breast cancer cells.

Effects of pre-miRNAs and *FoxM1* siRNA Transfection on Selective Proteins

Our results, as described above, revealed an effect of DIM plus Herceptin on *miR-200* and *FoxM1* expression individually. We were now interested in establishing a mechanism for this observation. Since miRNAs modulate the expression of their target genes and *FoxM1* is a predicted target of *miR-200*, we asked the question whether ectopic expression of *miR-200* can affect the expression of *FoxM1*. Such an observation will suggest that DIM/Herceptin-mediated regulation of *miR-200* is important for the eventual regulation of *FoxM1*, which ultimately defines the aggressiveness, and possibly, the drug resistance of breast cancer cells. SKBR3 and MDA-MB-468 cells were transfected with either pre-miR-200 or *FoxM1* siRNA followed by DIM and Herceptin treatment for 48 hours. In fig. 5A, it is clear that in SKBR3 cells, pre-miR-200, DIM plus pre-miR-200, as well as Herceptin plus pre-miR-200, were found to down-regulate *FoxM1* and the combination was found to be significantly more effective. On the other hand, the expression of *pAkt* and *NF- κ B p65* was further reduced with both the transfections (Fig. 5). We did not observe any effect on *FoxM1*, *NF- κ B p65* and *pAkt* in MDA-MB-468 treated with pre-miR-200, DIM plus pre-miR-200, as well as Herceptin plus pre-miR-200 individually. However, combination treatment down-regulates those genes in MDA-MB-468 breast cancer cells although more significant effects were observed in SKBR3. Conversely, transfection with *FoxM1* siRNA in both SKBR3 and MDA-MB-468 cell lines showed inhibitory effects on *FoxM1*, *NF- κ B p65* and *pAkt*, although more significant effects were observed in SKBR3 suggesting that DIM enhances Herceptin

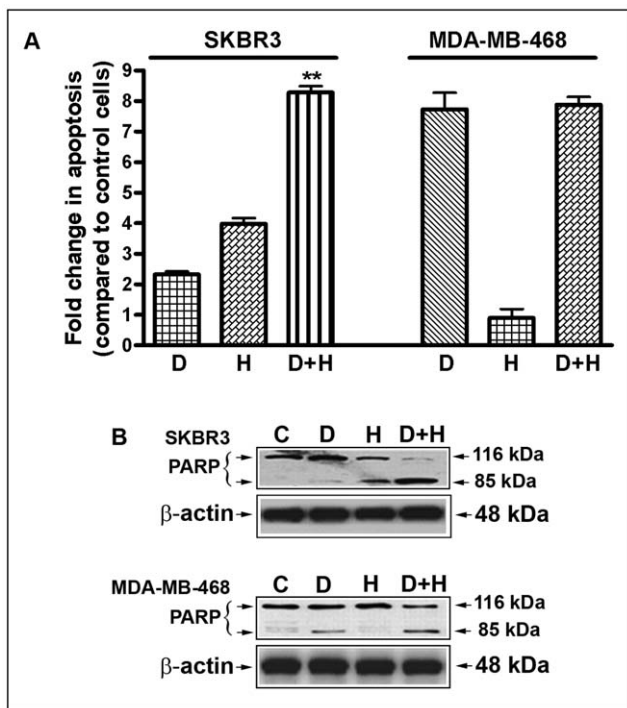


Figure 2. Effects of DIM and Herceptin on apoptosis. (A) Induction of apoptosis in SKBR3 and MDA-MB-468 cells treated with 15 μ M of DIM, 0.75 μ g/ml of Herceptin, and their combination. Fold changes in DIM and Herceptin-treated SKBR3 and MDA-MB-468 cells relative to untreated cells are shown. (B) PARP cleavage assay showed that combination treatment with DIM and Herceptin induced significantly greater apoptosis. The p values (**, $P < 0.01$) represent comparisons between cells treated by either of the drugs and their combinations by using the paired t test. C, Control; D, DIM (15 μ M/L DIM); H, Herceptin (0.75 μ g/ml). doi:10.1371/journal.pone.0054657.g002

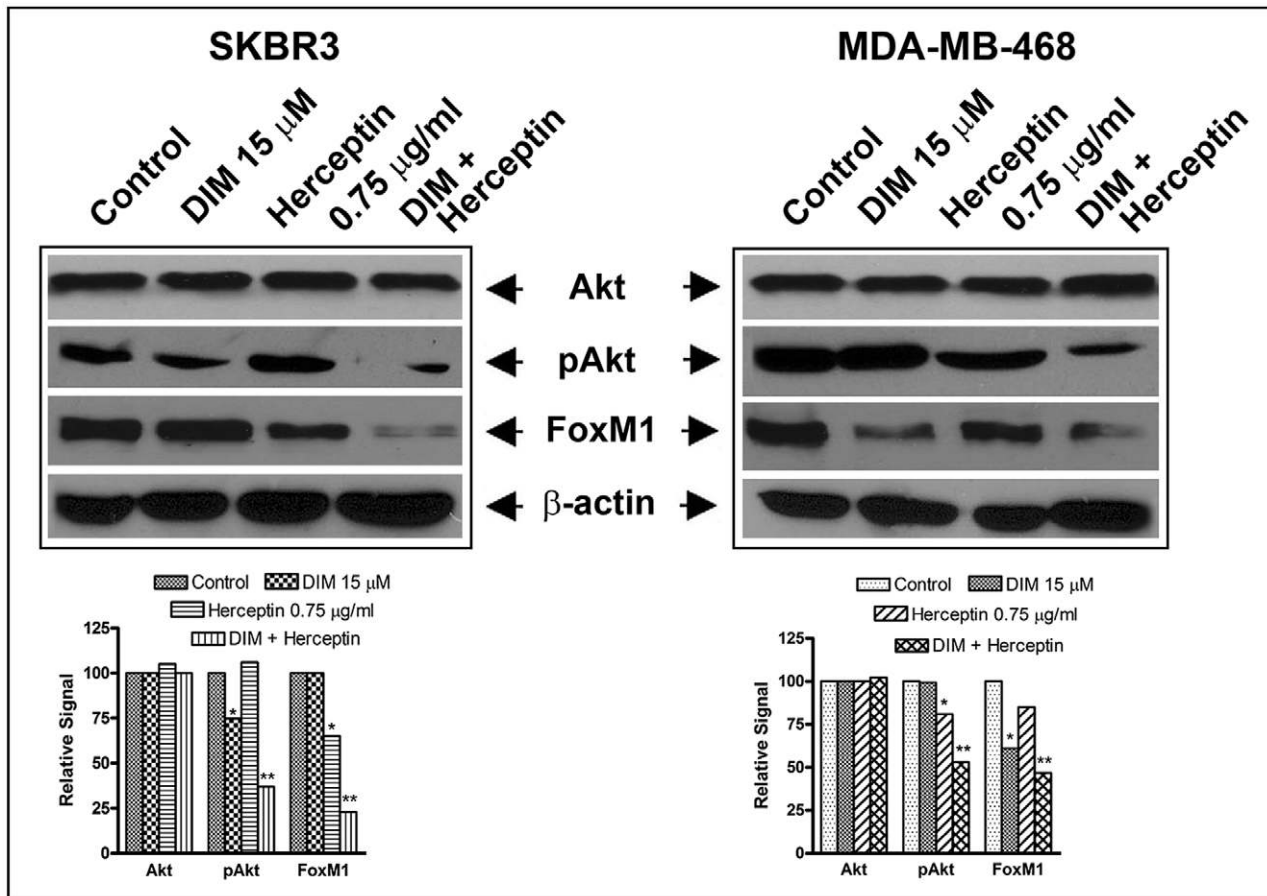


Figure 3. Effects of DIM and Herceptin on selective proteins. Expression of *FoxM1*, *Akt*, *pAkt* and *NF- κ B p65*, and β -actin in SKBR3 and MDA-MB-468 cell lines treated with 15 μ M DIM, 0.75 μ g/ml Herceptin or the combination for 72 hours. The bar graphs at the bottom in each case are based on quantitative values of bands of interest normalized to β -actin. *, $P < 0.05$ and **, $P < 0.01$ relative to control. doi:10.1371/journal.pone.0054657.g003

activity in breast cancer cells, but significant effect was found in *HER-2/neu*-expressing breast cancer cells. Collectively, these findings clearly suggest that DIM and Herceptin are capable of down-regulating the expression of *FoxM1*, which is normally high in malignant tumors, and upregulating the expression of *miR-200*.

Discussion

DIM is a major *in vivo* acid-catalyzed condensation product of indole-3-carbinol (I3C) and a plant-derived, non-toxic, dietary agent that selectively kills breast cancer cells without affecting normal cells [15,38–40]. Previously we have shown that I3C can inhibit breast cancer cell growth in a dose dependent manner in *HER-2/neu* over-expressing and in normal *HER-2/neu*-expressing cells by up-regulating *Bax*, down-regulating *Bcl-2* [38]. We also reported that DIM significantly inhibited the growth of breast tumor without associated toxicity [13–16,19]. Another study showed that DIM in combination with paclitaxel can inhibit cell proliferation growth by decreasing *Bcl-2* in *HER-2/neu* human breast cancer cells [41]. This report also showed that DIM alone can decrease the activation of the *HER-2/neu* receptor and the combination decreases the activation of *ERK1/ERK2* [41]. DIM sensitizes pancreatic tumor cells to targeted therapeutic agents such as EGFR inhibitor Erlotinib, potentiating apoptotic effect through altered regulation of EGFR signaling [42]. It has been shown that DIM or isoflavones could function as miRNA

regulators leading to the reversal of EMT phenotype [43]. Recently, we have shown that DIM could enhance the therapeutic efficacy of Taxotere in breast and prostate cancer cells [13,14,19] suggesting that DIM can sensitize cancer cells to chemotherapeutic agents. These findings led us to hypothesize that the combining effect of DIM and Herceptin may result in an enhanced antitumor response, which could be a useful approach for the treatment of human breast cancers, particularly *HER-2/neu*-expressing breast cancer patients.

Current Herceptin-based combination therapies involving conventional chemotherapeutic drug increase response rates, time to disease progression, and survival of patients with high level of *HER-2/neu* [44]. However, these treatments are associated with several toxicities [44]. A majority of cancers that initially respond to Herceptin, develop metastatic disease within 12 months [3,45–47]. Neither Herceptin's mechanisms of action nor those of disease progression are known, but they likely involve *HER-2/neu* and its downstream signaling pathways [4,47]. Therefore, identifying the mechanisms responsible for aggressiveness that could be suppressed by DIM plus Herceptin is important for the development of new therapeutic strategies. Our data demonstrate the efficacy of DIM to increase the effectiveness of Herceptin by significant killing of breast cancer cells in *HER-2/neu* expressing breast cancer cells.

FoxM1 belongs to a family of proteins that is evolutionary conserved and which is marked by the presence of DNA-binding

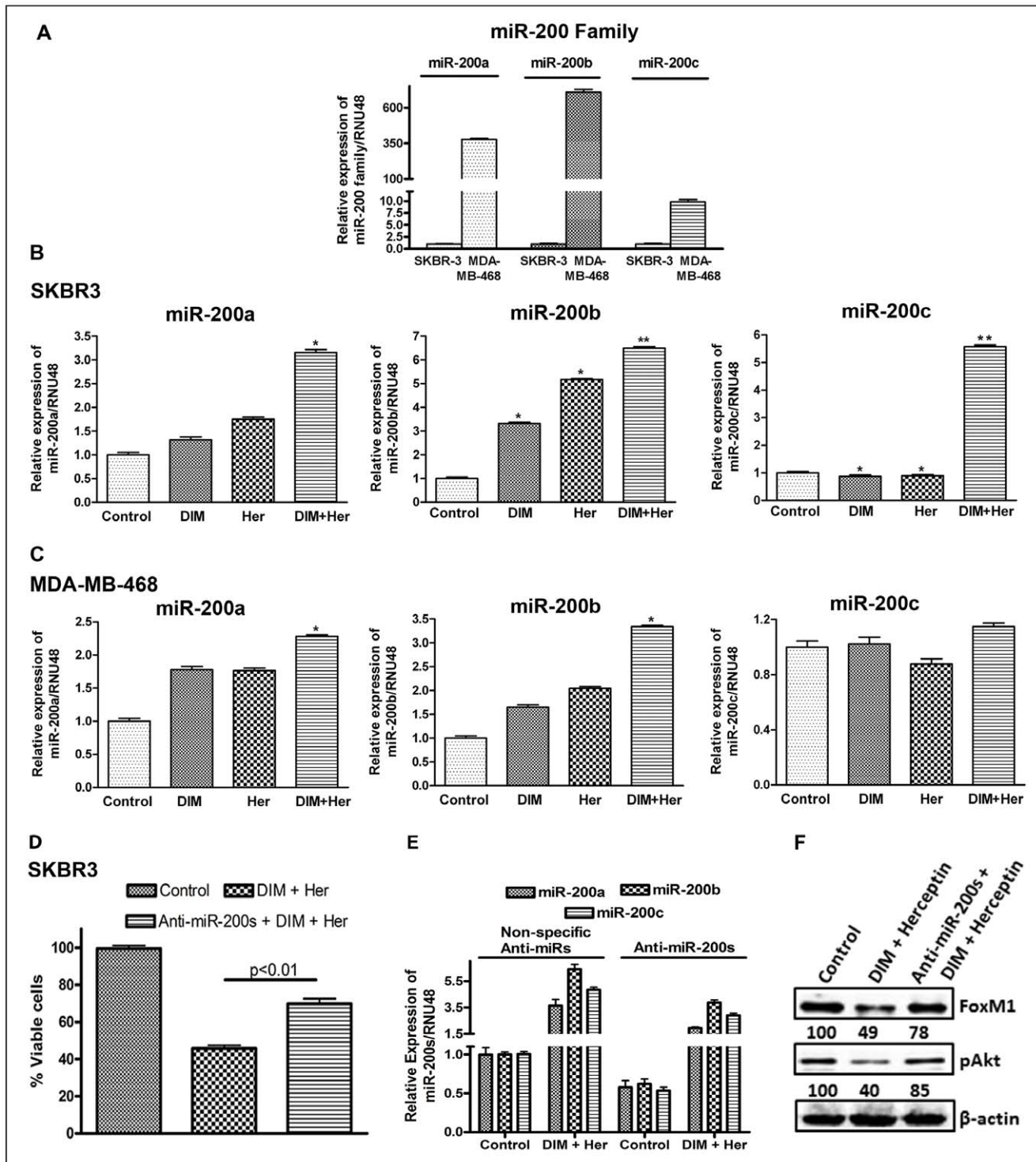


Figure 4. Effects of DIM and Herceptin on *miR-200* family. (A) Comparative expression analysis of *miR-200* in SKBR3 and MDA-MB-468 cells by real-time miRNA RT-PCR. (B and C) Relative expression analysis of *miR-200* in SKBR3 and MDA-MB-468 cells treated with 15 μ M DIM and 0.75 μ g/ml Herceptin or their combinations for 24 h. (D) Effect of anti-*miR-200s* (anti-*miR-200a*+anti-*miR-200b*+anti-*miR-200c*) on cell proliferation of SKBR3 cells treated with 15 μ M DIM and 0.75 μ g/ml Herceptin for 72 hours, as measured by MTT assay. (E) Efficacy of anti-*miR-200s* transfections as well as the effect of DIM plus Herceptin (15 μ M DIM and 0.75 μ g/ml Herceptin for 72 hours) on expression levels of anti-*miR-200s* (*miR-200a*/*miR-200b*/*miR-200c*) was evaluated by real time RT-PCR. (F) Effect of anti-*miR-200s* (anti-*miR-200a*+anti-*miR-200b*+anti-*miR-200c*) on expression of *FoxM1* and *pAkt* in SKBR3 cells treated with 15 μ M DIM and 0.75 μ g/ml Herceptin for 72 hours, as determined by western blotting. The numbers represent percent of corresponding control normalized to β -actin levels. Her, Herceptin; anti-*miR-200s*, anti-*miR-200a*+anti-*miR-200b*+anti-*miR-200c*. *, $P < 0.05$ and **, $P < 0.01$ relative to control.

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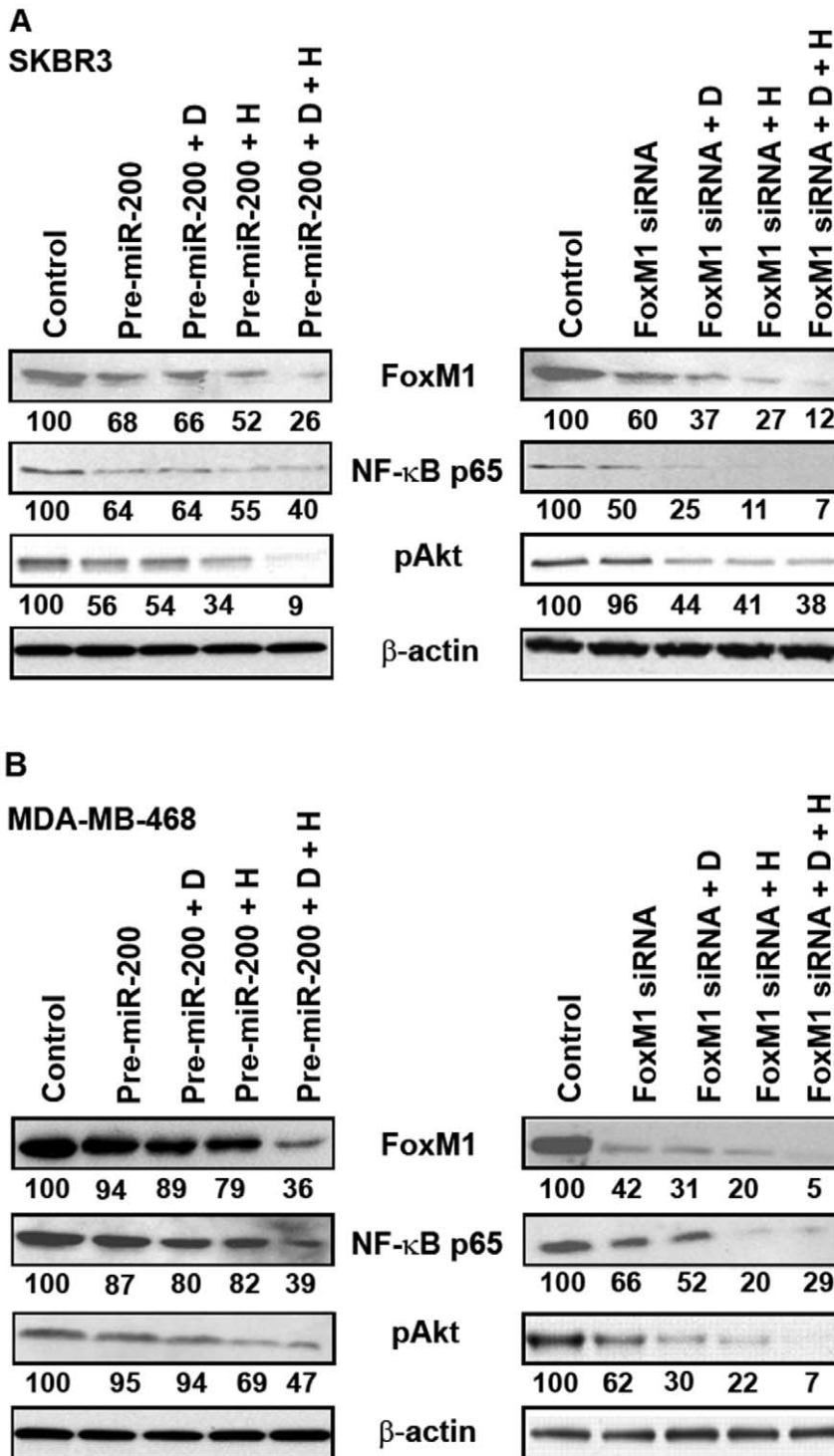


Figure 5. The expression of *FoxM1*, *Akt*, *pAkt* and *NF-κB p65* in breast cancer cells lines. Expression of *FoxM1*, *Akt*, *pAkt* and *NF-κB p65* in SKBR3 and MDA-MB-468 cells after transfection with pre-miRNAs (pre-miR-200a+pre-miR-200b+pre-miR-200c) and FoxM1 siRNA followed by 15 μM of DIM and 0.75 μg/ml of Herceptin for 48 h. D, DIM; H, Herceptin. The numbers represent percent of corresponding control normalized to β-actin levels.

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domain called 'forkhead box'. The various members of Fox family have been implicated in the progression of many cancers, including those of breast, liver, prostate, brain and lung through their ability to drive cell cycle progression and evasion of growth arrest [31]. *FoxM1* is a key regulator of transition from G1 to S

phase as well as for the progression to mitosis. Loss of *FoxM1* expression has been connected to mitotic spindle defects and accumulation of cells in mitosis, which leads to a mitotic catastrophe. Using microarray studies, we first identified *FoxM1* as a target of DIM [16]. The consequence of *FoxM1* expression on

the cell growth, clonogenicity migration and invasion was later evaluated [31]. More recently, we showed that DIM can down-regulate *FoxM1* in multiple breast cancer cells leading to a potentiation of Taxotere action [19].

The miRNAs have emerged as principal regulators of various biological and pathologic processes. They are small (19–24 nucleotides) non-coding RNA molecules that down-regulate gene expression by interacting with sequences located in the 3' untranslated region (UTR) of multiple target mRNAs, resulting in either translational repression or degradation of mRNAs. The regulation of oncogenes/tumor suppressor genes by miRNAs is increasingly being realized as a key step in the progression of human malignancies. It has been shown that miRNAs play an important role in modulating multiple cellular pathways, including cell proliferation, differentiation, and apoptosis, and thus may function as oncogenes or tumor suppressor genes [20,48,49]. In particular, decreased expression of miRNA-200 (*miR-200a*, *miR-200b* and *miR-200c*) has been reported in breast cancer [50]. Furthermore, *miR-200* family members are associated with resistance to several chemotherapy drugs: docetaxel in non-small cell cancer cells cisplatin in breast cancer cells and gemcitabine in cholangiocarcinoma cells [51–53]. In our study we found that DIM in combination with Herceptin can upregulate *miR-200* expression, a master regulator of genes expression and intimately involved in breast cancer. As a direct proof of mechanistic involvement of *miR-200* in DIM plus Herceptin action, we demonstrated that antagonizing miR-200 significantly attenuated the cytotoxic effect of DIM plus Herceptin. *miR-200* family is particularly involved in the invasion and metastasis of breast cancer cells through its involvement in the regulation of epithelial-mesenchymal transition (EMT) [21]. Thus, effective up-regulation of *miR-200* family by DIM can potentially help overcome the aggressive phenotype of breast cancer cells, a phenotype that is hallmark of metastatic as well as drug-resistant breast cancers. Our results suggest that DIM and Herceptin could affect miRNAs, which is likely to aid in designing novel therapies for breast cancer.

On the other hand, post-transcriptional regulation of gene expression by miRNA has recently attracted major interest among researchers in relation to its involvement in cancer development [48]. Every miRNA potentially regulates the expression of numerous protein coding genes (tens to hundreds), but it has become increasingly clear that not all miRNAs are equally important. *FoxM1* is a transcription factor that is important for cellular processes in carcinogenesis. By increasing expression of *FoxM1*, these miR-200s could promote oncogenesis and progression of various carcinomas, and contribute to chemotherapeutic resistance. However, whether *FoxM1*-targeting miR-200s are involved in progression of breast cancer has not yet been clarified. Consistent with our results, it was shown that down-regulation of *FoxM1* by treatment of cells with *FoxM1* siRNA and pre-miR-200s transfection decreased *FoxM1* expression. The implication of our data would be enormous because natural nontoxic agents could be useful for the down-regulation of *FoxM1* mediated via upregulation of *miR-200*, and our data further suggest that such strategy could in fact be useful for sensitization of drug-resistant breast cancer cells to other agents that are not very effective by themselves, such as Herceptin. The down-regulation of *FoxM1* by DIM and Herceptin would decrease *Akt* phosphorylation and *NF-κB p65*, which would likely contribute to the inhibition of cell growth and induction of apoptosis, and such effects could be exploited in a preclinical animal model in future studies.

In this study, we were puzzled by the observation that a combination of DIM and Herceptin was significantly better than DIM alone in MDA-MB-468 cells. As expected, Herceptin alone

had no to minimal activity in MDA-MB-468 cells. Since MDA-MB-468 cells do not express *HER-2/neu*, and Herceptin is very specific for HER2, MDA-MB-468 cells were rather insensitive to *HER-2/neu* alone for most of the parameters studied. In such a scenario we were expecting a combination of DIM and Herceptin to be no better than DIM alone. However, to our surprise, we observed the combination to be significantly better than either agent alone, in many assays. Herceptin, being a monoclonal antibody, is highly specific for *HER-2/neu* and though there are reports on its sensitization but such studies have only looked at *HER-2/neu* as its target. A careful survey of literature led us to couple of interesting reports. In one of these reports [54], which also used MDA-MB-468 cells, it was shown that knockdown of protein synthesis regulator eEF-2 leads to sensitization of MCF-7 as well as MDA-MB-468 cells to Herceptin. With little to no *HER-2/neu* expression, none of these cell lines is expected to respond to Herceptin. Deregulation of a single gene made these cells sensitive to Herceptin, which clearly demonstrates that individual cellular events can make even *HER-2/neu* negative cells sensitive to Herceptin. In present study, inhibition of *FoxM1* by DIM might be the cellular event leading to sensitization to Herceptin treatment (as observed by significantly higher activity in combinational treatment). Also, although Herceptin is believed to be very specific for *HER-2/neu*, there is evidence to indicate non-specific side effects of this drug in *HER-2/neu* negative cells [55]. This report looked at non-specific effects of Herceptin on miRNAs in MCF-7 and MDA-MB-231 cells and reported significant upregulation as well as down-regulation of several miRNAs. While miR-200s were not specifically recognized in this particular study, several other miRNAs such as let-7 family miRNAs and *miR-34a* were found to be down-regulated by Herceptin in *HER-2/neu* negative cells. These miRNAs play very similar role as *miR-200* in the regulation of EMT. Few other miRNAs mentioned in the study, such as *miR-148a* and *miR-16*, have also been linked to similar biological functions as miR-200. Therefore, although *let-7s*, *miR-16*, *miR-34a*, *miR-148a* and some other miRNAs were identified as non-specific targets of Herceptin in MCF-7 and MDA-MB-231 cells, it is possible that miR-200s are the non-specific targets in MDA-MB-468 cells. These reports lend credibility to our novel observations but clearly more mechanistic studies need to be performed in future to fully understand this action of Herceptin, which is beyond the scope of our current study.

In conclusion, our results suggest that DIM enhances the efficacy of Herceptin through upregulation of miR-200s in breast cancer cells. This is accompanied by down-regulation of *FoxM1*, *Akt* and *NF-κB p65* which needs further mechanistic evaluations. Our results could be exploited for the development of a novel therapeutic strategy to treat breast cancer, particularly *HER-2/neu*-expressing breast cancer using the combination of DIM plus Herceptin in the immediate future. These findings also highlight the potential usefulness of DIM, a non-toxic chemoprotective agent for treating *HER-2/neu*-expressing breast cancer. We have also provided a direct correlation between miR-200 and DIM plus Herceptin action, however, further in-depth molecular investigations are needed to fully address the mechanisms by which DIM potentiates Herceptin effects.

Author Contributions

Conceived and designed the experiments: A. Ahmad KMWR. Performed the experiments: A. Ahmad SA A. Ahmed A. Ali KMWR. Analyzed the data: A. Ahmad SA A. Ahmed A. Ali KMWR. Contributed reagents/materials/analysis tools: A. Ahmad SA A. Ahmed A. Ali KMWR. Wrote the paper: A. Ahmad AR WAS KMWR.

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