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Full length paper

EGFR inhibitors in combination with cyclopamine as chemotherapeutic strategy for treating breast cancer

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ABSTRACT

Purpose: To determine whether combination therapy targeting sonic hedgehog (HH) and epidermal growth factor receptor (EGFR) pathways can inhibit breast cancer growth.
Methods: Cell viability was determined by MTT assay, apoptosis assessed using flow cytometry and endogenous expression of Gli-1, SHH and SMO determined by real time RT-PCR.
Results: Higher endogenous expression of the hedgehog signaling genes Gli-1, SHH and SMO was observed in MCF-7 cells compared to MDA-MB-231 cells. In contrast, MDA-MB-231 cells have higher endogenous expression of EGFR. Among the two EGFR inhibitors studied, afatinib was more potent than gefitinib regardless of cell line and exposure time and cyclopamine more potently inhibited breast cancer cells than vismodegib. As determined using combination index analysis and three-dimensional response surface methodology, simultaneous combination of cyclopamine and afatinib or gefitinib was synergistic at selected concentrations and mixing ratios. Regarding exposure schedules, only sequential treatment of afatinib followed by cyclopamine exhibited a synergistic effect (CI = 0.24) in MDA-MB-231 cells. Also, combination of cyclopamine and afatinib or gefitinib was more potent in inducing apoptosis compared to monotherapy in MDA-MB-231 cells. Additionally, combination of cyclopamine and gefitinib more effectively downregulated Gli-1 expression in MDA-MB-231 breast cancer cells compared to monotherapy.
Conclusions: Combination of cyclopamine and afatinib or gefitinib leads to a supra-additive inhibition of MDA-MB-231 and MCF-7 cell proliferation and was found to be dose, time, cell line and schedule dependent. Also, combination therapy more effectively induced apoptosis, inhibited cell migration and downregulated Gli-1 expression compared to monotherapy in breast cancer cells.

1. Introduction

Breast cancer is one of the most common malignancies affecting women in the United States and remains the second-leading cause of cancer related mortality amongst women worldwide [1,2]. Because of the threat of breast cancer to women's health in the US and the world at large, tremendous efforts have been devoted to identifying and understanding key pathways that promote breast cancer cell proliferation. Additionally, attention has been focused on developing potent anticancer molecules that can interrupt these pertinent breast cancer pathways so as to mitigate morbidity and mortality.

One of the several pathways encountered in embryogenesis, homeostasis, stem cell maintenance, carcinogenesis, tumor proliferation and angiogenesis is the hedgehog (HH) signaling pathway [3]. This pathway is important because of its ability to inhibit apoptosis by increasing cyclin proteins, anti-apoptotic factors and decreasing fas pro-apoptotic genes [4]. The sonic hedgehog (SHH) signaling transduction pathway is one of the most well studied vertebrate pathways. Presence of Hh receptor patch (PTCH) 1 tumor suppressor gene and the activation of SHH pathway is strongly associated with clinical aggressiveness of breast carcinomas [5]. Furthermore, it has been shown that the binding of SHH to PTCH alleviates inhibition, which regulates the expression of Gli protein transcription factors [6]. When there is no ligand in this pathway, the PTCH receptor is capable of inhibiting another downstream protein called smoothened (SMO). Loss-of-function mutations of PTCH, gain-of-function mutations of SMO and dysregulation of the Gli-1, Gli-2 and Gli-3 have been associated

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with tumor formation and maintenance in ovarian, oesophageal and prostate cancers [7–11]. It has been reported that over 40% of breast cancers overexpress Gli-1 [12–15]. Overexpression of Gli-1 is associated with the invasive potential of breast cancer tumors and reported to correlate with poor prognosis in patients [16–18]. Targeting the hedgehog pathway in breast cancer is still a challenge and several approaches including natural products, monoclonal antibodies and synthetic small molecules have been investigated. Presently, cyclopamine and vismodegib are two widely studied small molecule hedgehog antagonists which inhibit the hedgehog signaling pathway by binding to SMO. Although cyclopamine was identified before visomodegib, its clinical application has been hampered due to its poor bioavailability, short half-life, chemical instability and non-specific toxicity [19]. Vismodegib is a steroidal alkaloid cyclopamine that has antitumor activities and has been approved by the FDA for treatment of advanced basal cell carcinoma. However, it is contraindicated during pregnancy due to its teratogenicity, embryotoxicity and fetoxicity [20,21]. Improved understanding of the hedgehog pathway has led to the development a new class of antagonists such as GANT58 and GANT61 which block GLI transcriptional activity and hence target the hedgehog pathway downstream of SMO [22,23]. This therapeutic approach is particularly attractive for breast cancer which exhibit SMO mutations.

Epidermal growth factor receptor (EGFR) signaling pathway is another signaling pathway that plays a critical role in the regulation, growth, survival, proliferation, and differentiation of breast and other cancers [24]. This pathway consist of EGFR endocytosis followed by its degradation or recycling, small guanosine triphosphatase (GTPase)-mediated signal transduction such as mitogen-activated protein kinase (MAPK) cascade, phosphatidylinositol polyphosphate (PIP) signaling, cell cycle, and G protein-coupled receptor (GPCR)-mediated EGFR downstream transactivation via intracellular Ca^{2+} signaling [25]. Accumulating evidence suggests EGFR consists of free EGFR and the following complexes: EGF–EGFR, EGFR–TKI and EGF–EGFR–TKI. The EGF–EGFR complex is the form that is responsible for the EGFR pathway activation [26]. Also when activated, the signal transduction is started-up through RAS/MAPK and PI3K/AKT pathways which provokes cell proliferation and survival [27]. Matrix metalloproteinase-9 (MMP9), which is closely associated with human tumor invasion and metastasis, has been shown to be inhibited by EGFR signaling pathway blockade. The EGFR pathway is over expressed in all types of cancers, and 70–90% of those with *EGFR* overexpression have *EGFR* amplification [28–30]. In breast cancer such over expression of *EGFR* is a predictor of BRCA1 status and are highly correlated with poor prognosis [31]. The presence of downstream signaling proteins (e.g., src and Ras/MAPK) facilitates targeting with EGFR inhibitors, antibodies and vaccines [32,33]. It is also important to target EGFR because there is substantial evidence supporting the fact that EGFR overexpression is correlated with treatment resistance [34,35]. Afatinib and gefitinib are two molecules that effectively target the EGFR pathway by selectively inhibiting EGFR/HER1 and HER2 EGFR activity and play a pivotal role in tumor growth control.

Despite advances made in understanding hedgehog and EGFR signaling pathways and the development of potent antagonists, this success has yet to translate into improved patient outcome. A primary reason for this is that current therapies typically target a single oncogenic pathway. It is well known that combining anticancer drugs with unique mechanism of action, non-overlapping toxicities and targeting different distinct pathways typical result in better therapeutic effects [36,37]. Recently, Mimeault et al. demonstrated that combining gefitinib and cyclopamine more effectively inhibited prostate cancer cell proliferation and induced a higher apoptotic rate compared to monotherapy [38]. Also, combination of cyclopamine and gefitinib has been shown to result in a synergistic effect against L3.6pl pancreatic cancer cells but an additive effect against MIA PaCa-2 cells. Caspase 3/7 activity was also found to increase in pancreatic cells when exposed to combination therapy compared to single drug treatment [39].

In this study, we investigated the anti-proliferative and apoptotic effects of two EGFR inhibitors (afatinib and gefitinib) and two hedgehog inhibitors (vismodegib and cyclopamine). Our ultimate aim is to establish clinically relevant combinations, therefore we determined whether simultaneous and sequential combination of afatinib or gefitinib and cyclopamine was synergistic, additive or antagonistic. Additionally, we endeavored to identify drug combinations and mixing ratios which are potentially synergistic combinations for treating breast cancer.

2. Materials and methods

2.1. Materials

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and stored in liquid nitrogen. Dulbecco's modified Eagle medium (DMEM), TrypLE Express and antibiotic–antimycotic were obtained from Life Technologies (Carlsbad, CA). EGFR inhibitors (afatinib, axitinib and gefitinib) and hedgehog inhibitors (vismodegib and cyclopamine) were purchased from LC-Laboratories (Woburn, MA). All other reagents were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise stated and were used as received.

2.2. Cell culture

MDA-MB-231 and MCF-7 breast cancer cell lines were recovered from liquid nitrogen and maintained in DMEM supplemented with 10% FBS and 1% antibiotic–antimycotic. Cells were incubated with complete medium in a humidified incubator of 5% CO_2 at 37 °C. Cells were sub-cultured every 3–4 days to maintain exponential growth. For experiments, cells were seeded in 96-well plates at a density of 2×10^4 of viable cells per well or 6-well plates at a density 4×10^5 of viable cells per well following counting using a countess automated cell counter (Life Technologies, Carlsbad, CA) and incubated for 24, 48 or 72 h.

2.3. In vitro cell viability assay

Stock solutions of afatinib, gefitinib, vismodegib and cyclopamine were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at −20 °C. After cells had attached to the growth surface of the well plate, they were treated with various EGFR inhibitors (afatinib, gefitinib) or hedgehog inhibitor (vismodegib, cyclopamine) at various concentrations (0–100 μM) and for 24 and 72 h. For combination therapy, cells were simultaneously treated with a specified EGFR inhibitor and a specified hedgehog inhibitor. In this instance, concentration of EGFR inhibitors used ranged from 0 to 25 μM while concentration range of HH inhibitors was 1–25 μM. At the end of treatment, 20 μL of MTT (5 mg/mL) was added to each well and incubated for 3–4 h. The residual formazan crystals were solubilized with 200 μL DMSO and analyzed using a microplate reader recording absorbance values at a test wavelength of 560 nm. Cell viability for a given concentration was expressed as a percentage of the intensity of controls.

2.4. Assessment of combination effects

The anticancer effect of EGFR inhibitor and hedgehog inhibitor combination therapy was evaluated using two methods: isobologram analysis and the three-dimensional (3-D) model.

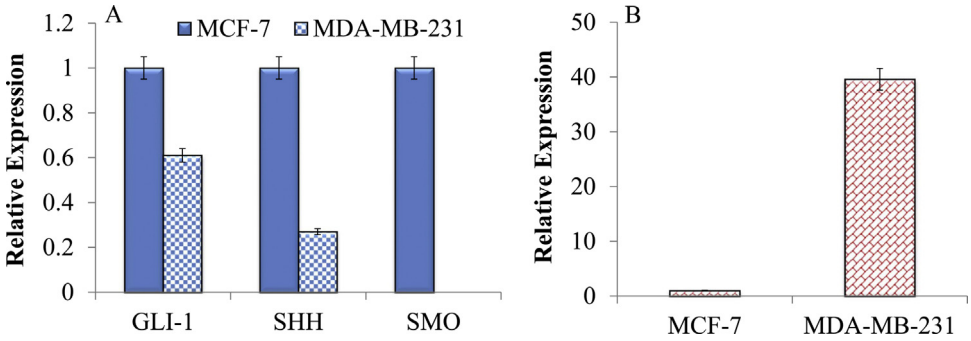

Fig. 1. Endogenous expression of hedgehog signaling components and EGFR in MDA-MB-231 and MCF-7 human breast cancer cell lines. mRNA levels of (A) Gli-1, SHH and SMO and (B) EGFR determined using real time RT-PCR.

(i) Isobologram analysis

The dose–effect interaction between EGFR inhibitors (afatinib, gefitinib) and hedgehog inhibitors (vismodegib and cyclopamine) at the point of IC_{50} was assessed to be synergistic, additive or antagonistic using the CompuSyn Program based on the median-effect method of Chou-Talalay [40]. Dose–response curves were plotted for the effects of EGFR Inhibitors and HH inhibitors on human breast cancer MDA-MB-231 and MCF-7 cell viability. From these curves, the combined drug IC_{50} values were determined for each curve. Specifically, MDA-MB-231 and MCF-7 cell lines cells were treated with EGFR inhibitors (afatinib, gefitinib) and HH inhibitors (vismodegib and cyclopamine) at concentrations of 0, 5, 10, 25 μM and 1, 5, 10 and 25 μM, respectively. Cell viability assessed using MTT assay resulting in the above-mentioned dose–effect curves. The combination index (CI) was calculated by Eq. (1):

$$CI = \frac{IC_{A,B}}{IC_A} + \frac{IC_{B,A}}{IC_B} \quad (1)$$

where IC_A and and IC_B are the concentrations of agent A (EGFR Inhibitor) and agent B (HH inhibitor) needed to produce a given level of effect alone, respectively. $IC_{A,B}$ and $IC_{B,A}$ are the concentrations required to produce the same effect when used in combination. The CI values were interpreted as follows: <1.0, synergism; 1.0, additive effect; >1.0, antagonism. Each experiment was performed in triplicates. The parameters IC_A and IC_B in Eq. (1) were obtained as follows: when a dose of (EGFR Inhibitor) (IC_A) was selected the incremental effect produced by adding (HH inhibitor) starting from 0 to 25 μM was assessed. The concentration of (HH inhibitor) that when combined with IC_A resulted in the given level of cell growth inhibition was designated IC_B.

(ii) Three-dimensional (3-D) Model

The potential of 3-D models to provide essential understanding of complex drug interactions has been described by Prichard and Shipman [41]. Three-dimensional surfaces of drug combinations were generated using response surface methodology (RSM) as follows. First, the cytotoxicity data obtained from monotherapy experiments were transformed to calculate additive interactions using the dissimilar site assumption of additivity equation (Eq. (2)) [41]:

$$Z = X + Y(1 - X) \quad (2)$$

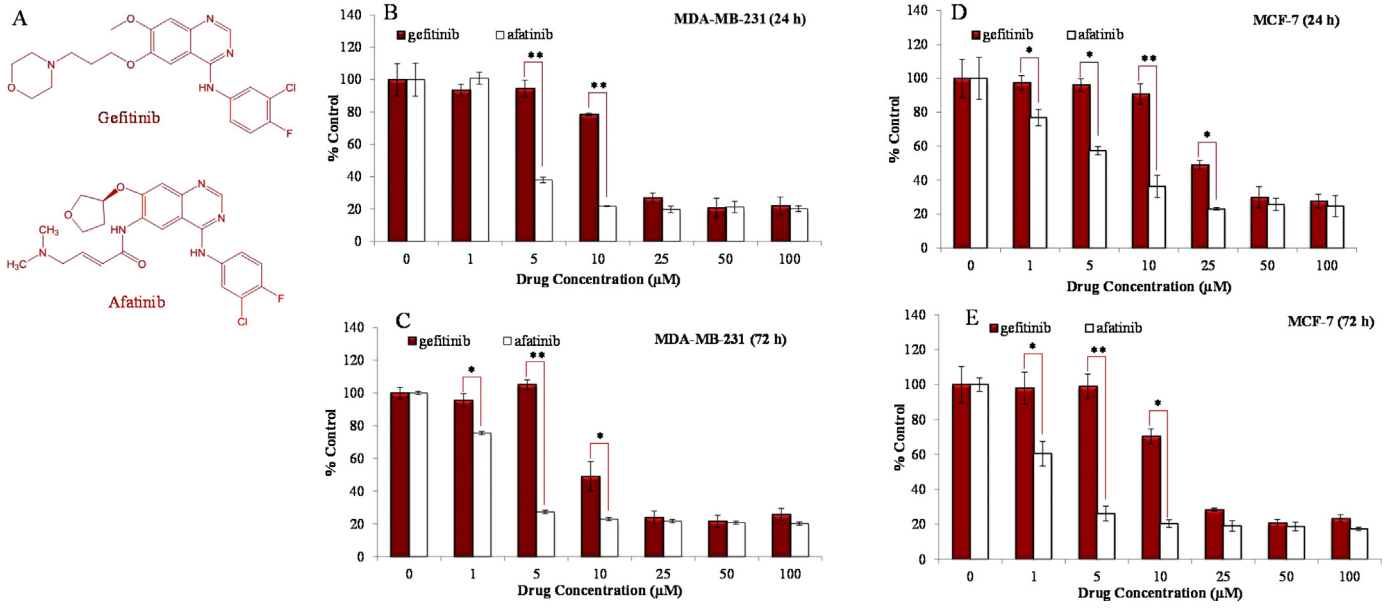

Fig. 2. Anticancer effect of EGFR inhibitors and hedgehog inhibitors on breast cancer cells. (A) Chemical structures of gefitinib and afatinib. (B and C) IC_{50} of gefitinib and afatinib in MDAMB-231 cells were calculated following treatment with 0–100 μM of drug for 24 h and 72 h. (D and E) IC_{50} of gefitinib and afatinib in MCF-7 cells were calculated following treatment with 0–100 μM of drug for 24 h and 72 h. (F) Chemical structures of vismodegib and cyclopamine. (G and H) IC_{50} of vismodegib and cyclopamine in MDA-MB-231 cells were calculated following treatment with 0–100 μM of drug for 24 h and 72 h. (I and J) IC_{50} of vismodegib and cyclopamine in MCF-7 cells were calculated following treatment with 0–100 μM of drug for 24 h and 72 h. Cell viability was determined by MTT assay. Results are represented as the mean ± SD of triplicates. $^{*}p < 0.05$; $^{**}p < 0.01$ using Student's unpaired t test.

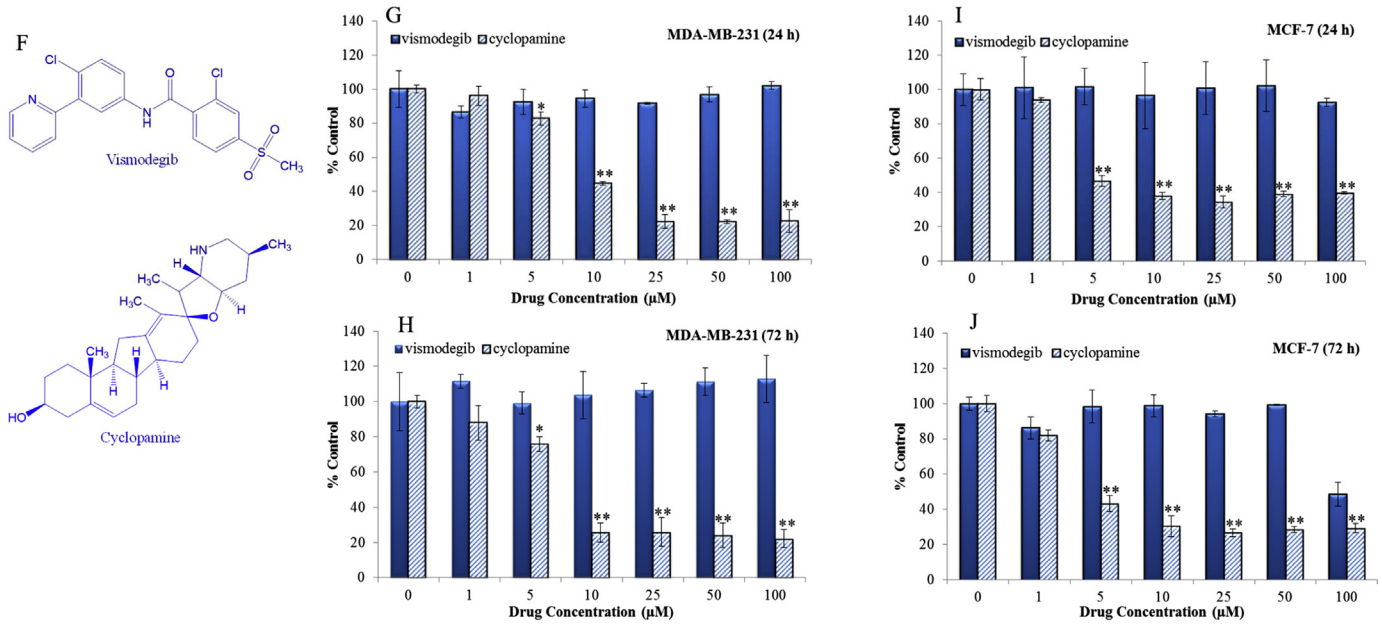

Fig. 2. (Continued)

Where *Z* is the total inhibition produced by the combination of drugs *A* and *B* and *X* and *Y* represent inhibition produced by drugs *A* and *B* alone, respectively. The result is a theoretical additive surface. Second, the theoretical additive surface is subtracted from the experimental 3-D surface obtained from cytotoxicity data of combination therapy. The required calculations were performed in Microsoft™ Excel (Microsoft Corporation, Redmond, WA). Finally, the difference between the experimental 3-D surface and the theoretical additive surface was imported from Microsoft Excel into Table Curve 3D (Systat Software Inc., San Jose, CA) to plot the data.

2.5. Analysis of apoptosis using flow cytometry

MDA-MB-231 cells were harvested using TrypLE Express following treatment of afatinib, gefitinib and cyclopamine alone or in combination. Cell density was adjusted to 1×10^6 cells/mL after which 1 μL of Hoechst 33,342 and 1 μL of propidium iodide (PI) was added to each group and incubated on ice for 30 min. Stained cells were subsequently subjected to flow cytometry and subsequently analyzed using FCS Express 5.

2.6. Cell migration assay

MDA-MB-231 cells were grown to 70% confluence in six well plates and three parallel wounds made using pipette tip. Micrographs of each wound were capture using an inverted microscope and used as a reference point. Cells were treated with cyclopamine (10 or 20 μM) or gefitinib (10 or 20 μM) alone or in combination for 48 h after which cells were washed with ice cold 1× PBS and imaged under an inverted microscope.

2.7. Real-time RT-PCR

RNA extraction and real-time RT-PCR was performed as previously described [42]. Primers used are as follows: Gli-1: forward, 5′-GGG ATG ATC CCA CAT CCT CAG TC-3′; reverse, 5′-CTG GAG CAG CCC CCC CAG T-3′. SHh: forward, 5′-GAT GTC TGC TGC TAG TCC TCG-3′; reverse, 5′-CAC CTC TGA GTC ATC AGC CTG-3′. SMO: forward, 5′-GTT CTC CAT CAA GAG CAA CCA C-3′; reverse, 5′-CGA TTC TTG ATC TCA CAG TCA GG-3′. EGFR: forward, 5′-TCC TCT GGA GGC TGA GAA AA-3′; reverse, 5′-GGG CTC TGG AGG AAA AGA AA-3′. β-actin: forward, 5′-AAA TCT GGC ACC ACA CCT TC-3′; reverse, 5′-CAG AGG CGT ACA GGG ATA GC-3′.

3. Results

3.1. Endogenous expression of hedgehog signaling components and EGFR in MCF-7 and MDA-MB-231Cells

We first examined the endogenous mRNA expression of hedgehog signaling components (Gli-1, SMO and SHH) and EGFR in MCF-7 and MDA-MB-231 cells using real time RT-PCR. From Fig. 1A, expression of hedgehog signaling components in MCF-7 was significantly higher compared to MDA-MB-231. Specifically, Gli-1 and SHH expression were 1.5 and 4 times more in MCF-7 cells compared to MDA-MB-231, respectively. Interestingly, no detectable expression of SMO was observed in MDA-MB-231 cells. In

Table 1
IC_{50} values of anticancer drugs tested on human breast cancer cell lines MCF-7 and MDA-MB-231.

Cell line	Drug	Exposure time (h)	IC_{50} (μM)
MCF-7	Gefitinib	24	29.28
		72	16.50
	Afatinib	24	5.92
		72	1.60
	Cyclopamine	24	6.04
		72	4.54
	Vismodegib	24	BCR[a]
		72	98.16
MDA-MB-231	Gefitinib	24	16.80
		72	10.26
	Afatinib	24	5.26
		72	2.60
	Cyclopamine	24	12.75
		72	15.94
	Vismodegib	24	BCR[a]
		72	BCR[a]

MCF-7 or MDA-MB-231 human breast cancer cells were exposed to drugs at concentrations ranging from 0 to 100 μM for 24 h or 72 h and percent viability calculated. Each data point represents the mean of three independent experiments.
[a] Beyond concentration range examined.

contrast, expression of EGFR in MDA-MB-231 was approximately 40-fold more than that of MCF-7 cells (Fig. 1B).

3.2. Effect of gefitinib and afatinib on MCF-7 and MDA-MB-231Cell proliferation

We next characterized the ability of two EGFR inhibitors: gefitinib and afatinib (Fig. 2A) to treat breast cancer by determining their IC_{50} values in MCF-7 (early stage) and MDA-MB-231 (advanced stage) breast cancer cells. Anticancer effect of gefitinib and afatinib was found to be dose- and time- dependent regardless of cell type (Fig. 2B–E) and IC_{50} values are summarized in Table 1. Our studies revealed afatinib to be most potent in inhibiting cell proliferation with IC_{50} values of 5.26 μM and 2.60 μM in MDA-MB-231 cells at 24 and 72 h, respectively. In MCF-7 cells, IC_{50} values for afatinib were 5.92 μM and 1.60 μM at 24 and 72 h, respectively. In contrast, gefitinib had IC_{50} values of 16.80 μM and 10.26 μM in MDA-MB-231 cells at 24 and 72 h, respectively, while its IC_{50} values in MCF-7 cells was 29.28 μM and 16.50 μM at 24 and 72 h, respectively.

3.3. Cyclopamine more potent than vismodegib in inhibiting MCF-7 and MDA-MB-231cell growth

To select a suitable hedgehog inhibitor for use in combination with afatinib or gefitinib, we determined the anticancer effect of cyclopamine and vismodegib (Fig. 2F) on breast cancer cell proliferation by examining their IC_{50} values in MCF-7 and MDA-MB-231 cells (Table 1). From Fig. 2G–J, cyclopamine was more potent than vismodegib in inhibiting cancer cell proliferation regardless of cell type. Specifically, IC_{50} values for cyclopamine in MDA-MB-231 cells were 12.75 μM at 24 h and 7.16 μM at 72 h while its IC_{50} values in MCF-7 cells were 6.04 μM and 4.54 μM at 24 h and 72 h, respectively. In contrast, vismodegib has no effect in MDA-MB-231 cells at 24 h and 72 h and MCF-7 cells at 24 h. However, IC_{50} for vismodegib in MCF-7 cells at 72 h was approximately 98.16 μM.

3.4. Effect of combination of cyclopamine and afatinib or gefitinib on MCF-7 and MDA-MB-231 cell proliferation

We investigated the simultaneous combination of cyclopamine and afatinib and cyclopamine and gefinitib in MCF-7 and MDA-MB-231 cells using the combination index and the response surface methodology. As shown in Fig. 3, combination of afatinib and cyclopamine was observed to be synergistic at lower drug concentrations at 24 h and antagonistic across all concentrations at 72 h regardless of cell line by the response surface method. In contrast, combination of gefitinib and cyclopamine was found to be synergistic at selected concetration at 24 and 72 h in both MCF-7 and MDA-MB-231 cells (Fig. 4). However, synergism was observed over a wider concentration range in MDA-MB-231 cells at 72 h for combination of gefitinib and cyclopamine.

Using the combination index approach, we evaluated simultaneous treatment of MCF-7 and MDA-MB-231 cells using afatinib or gefitinib and cyclopamine at a molar ratio of afatinib or gefitinib to

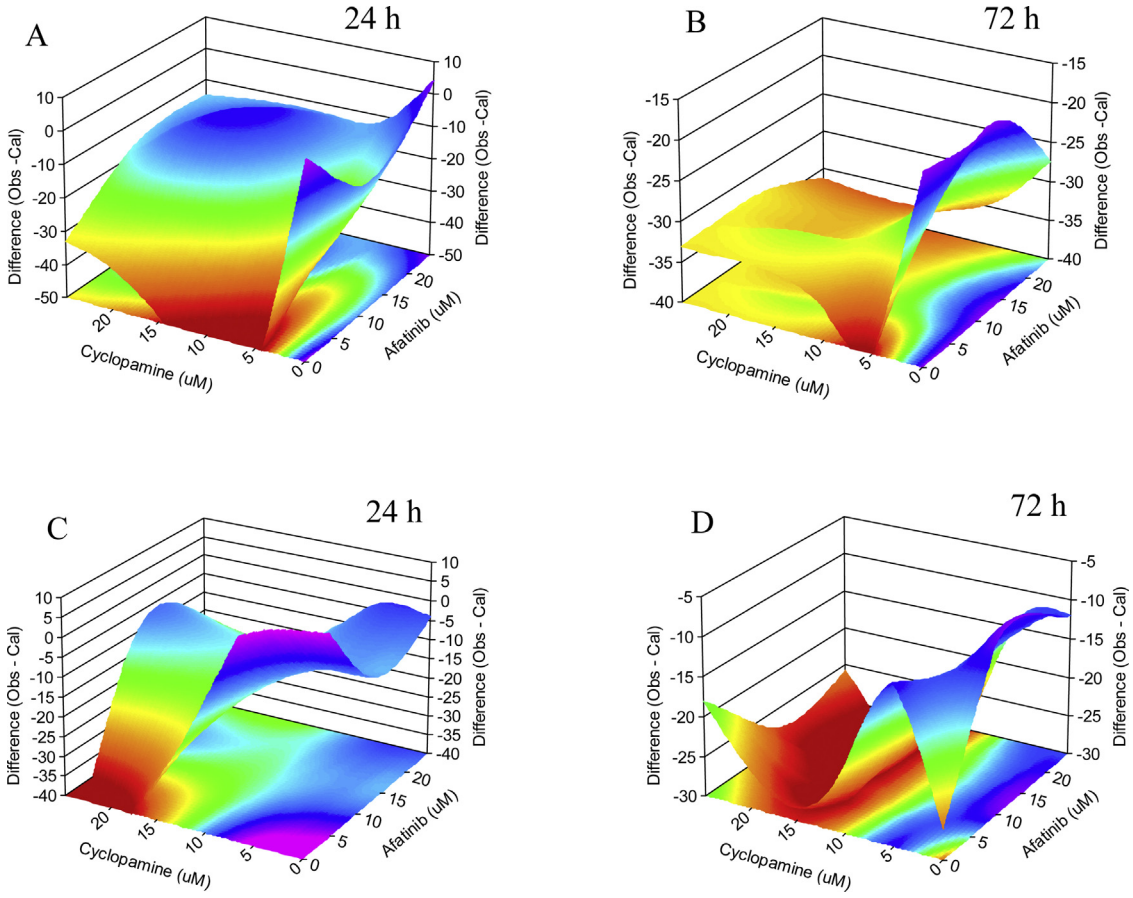

Fig. 3. Three-dimensional graphs representing anticancer interaction between afatinib and cyclopamine in human breast cancer cell lines. Cells were treated with afatinib and cyclopamine at concentrations ranging from 0 to 100 μM for 24 and 72 h and cell viability determined using MTT assay. MCF-7 cells treated for (A) 24 h and (B) 72 h. MDA-MB-231 cells treated for (C) 24 h and (D) 72 h. Portions of plot above zero on the difference axis indicate combinations that are synergistic while areas below zero are antagonistic and those equal to zero are additive.

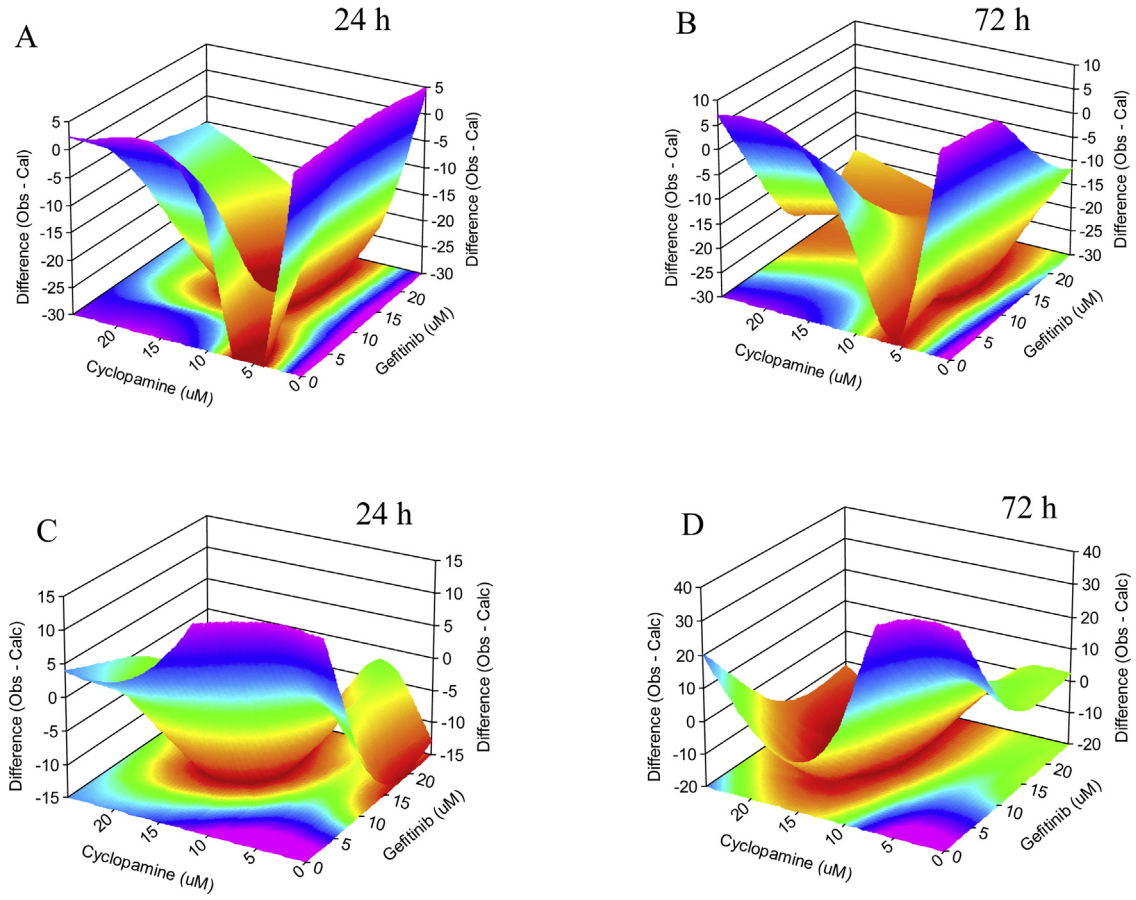

Fig. 4. Three-dimensional graphs representing anticancer interaction between gefitinib and cyclopamine in human breast cancer cell lines. Cells were treated with gefitinib and cyclopamine at concentrations ranging from 0 to 100 μM for 24 and 72 h and cell viability determined using MTT assay. MCF-7 cells treated for (A) 24 h and (B) 72 h. MDA-MB-231 cells treated for (C) 24 h and (D) 72 h. Portions of plot above zero on the difference axis indicate combinations that are synergistic while areas below zero are antagonistic and those equal to zero are additive.

cyclopamine of 5:1. Table 2 reveals combination of afatinib and cyclopamine to be synergistic at both 24 and 72 h in MDA-MB-231 cells and in MCF-7 cells at 72 h. However, this combination was antagonistic in MCF-7 cells at 24 h. On the other hand, combination of gefitinib and cyclopamine was synergistic at 24 and 72 h in MDA-MB-231 cells and antagonistic in MCF-7 cells at both time points.

3.5. Effect of exposure schedules of cyclopamine and afatinib or gefitinib on MCF-7 and MDA-MB-231 cell proliferation

We next investigated the effect of different exposure schedules of cyclopamine and afatinib and cyclopamine and gefinitib on MCF-7 and MDA-MB-231 cell growth using the isobologram method of Chou and Talalay. In this experiment, cells were exposed to either drug for 36 h for a total exposure time of 72 h. Cell viability was determined by MTT assay and the resulting dose–effect curves (Figs. 5 and 6) used to determine combination index. From Table 3, sequential treatment of afatinib followed by cyclopamine exhibited a synergistic effect (CI = 0.24) in MDA-MB-231 cells while it was antagonistic in MCF-7 cells. On the contrary, cyclopamine followed by afatinib was antagonistic regardless of cell line. Also, sequential administration of gefitinib and cyclopamine was observed to be antagonistic irrespective of cell line or exposure schedule.

Table 2
Combination index (CI_{50}) analysis of simultaneous treatment of human breast cancer cell lines MDA-MB-231 and MCF-7 for 24 and 72 h using afatinib or gefitinib and Cyclopamine. Molar ratio of afatinib or gefitinib to cyclopamine is 5:1.

Drug combination	Cell line	Time	CI_{50}	Interpretation
Afatinib + cyclopamine	MDA-MB-231	24	0.12	Synergism
Afatinib + cyclopamine	MDA-MB-231	72	0.06	Synergism
Afatinib + cyclopamine	MCF-7	24	1.80	Antagonism
Afatinib + cyclopamine	MCF-7	72	0.70	Synergism
Gefitinib + cyclopamine	MDA-MB-231	24	0.94	Synergism
Gefitinib + cyclopamine	MDA-MB-231	72	0.78	Synergism
Gefitinib + cyclopamine	MCF-7	24	1.48	Antagonism
Gefitinib + cyclopamine	MCF-7	72	1.57	Antagonism

The CI values are interpreted as follows: <1.0, synergism; 1.0 additive and >1.0, antagonism. Each experiment was done in triplicate.

3.6. Combination of cyclopamine and afatinib or gefitinib induces apoptosis and inhibits cell migration of MDA-MB-231 breast cancer cells

We next investigated the effect of combining EGFR inhibitors and hedgehog inhibitors on apoptosis. Specifically, MDA-MB-231 cells were treated with 10 μM of afatinib, gefitinib and cyclopamine alone or in combination for 72 h and the degree of apoptosis measured using flow cytometry. From Fig. 7A and B afatinib exhibited the highest rate of apoptosis (~30%) among the single drug treatment. In contrast, gefitinib and cyclopamine demonstrated similar levels of apoptosis (~10%) although the population of necrotic cells identified in the gefitinib treated group was double that of the cyclopamine treated group. Also, combination of afatinib and cyclopamine resulted in

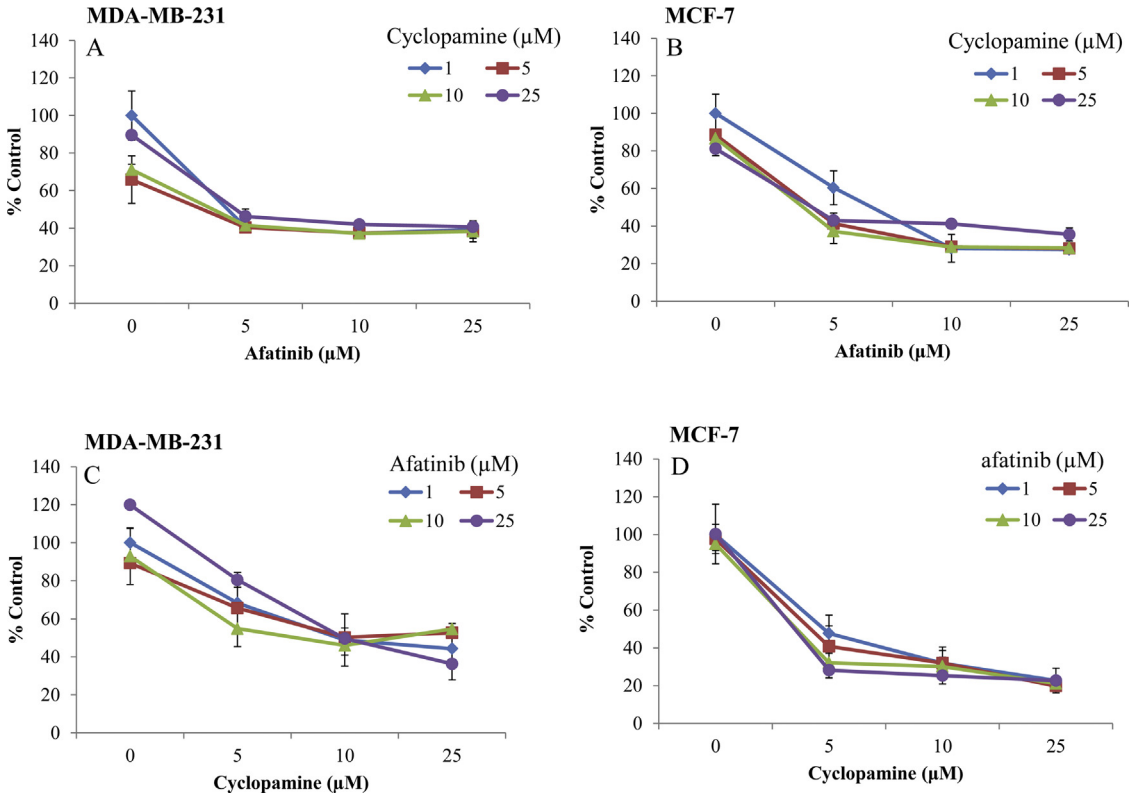

Fig. 5. Dose–effect curves for afatinib and cyclopamine combination in human breast cancer cells. Sequential exposure (36 h incubation periods to afatinib followed by cyclopamine in MDA-MB-231 and MCF-7 cells, respectively (A) and (B). Sequential exposure (36 h incubation periods) to cyclopamine followed by afatinib in MDA-MB-231 and MCF-7 cells, respectively (C) and (D). Cell viability was determined using MTT assay and plotted as a percentage of control.

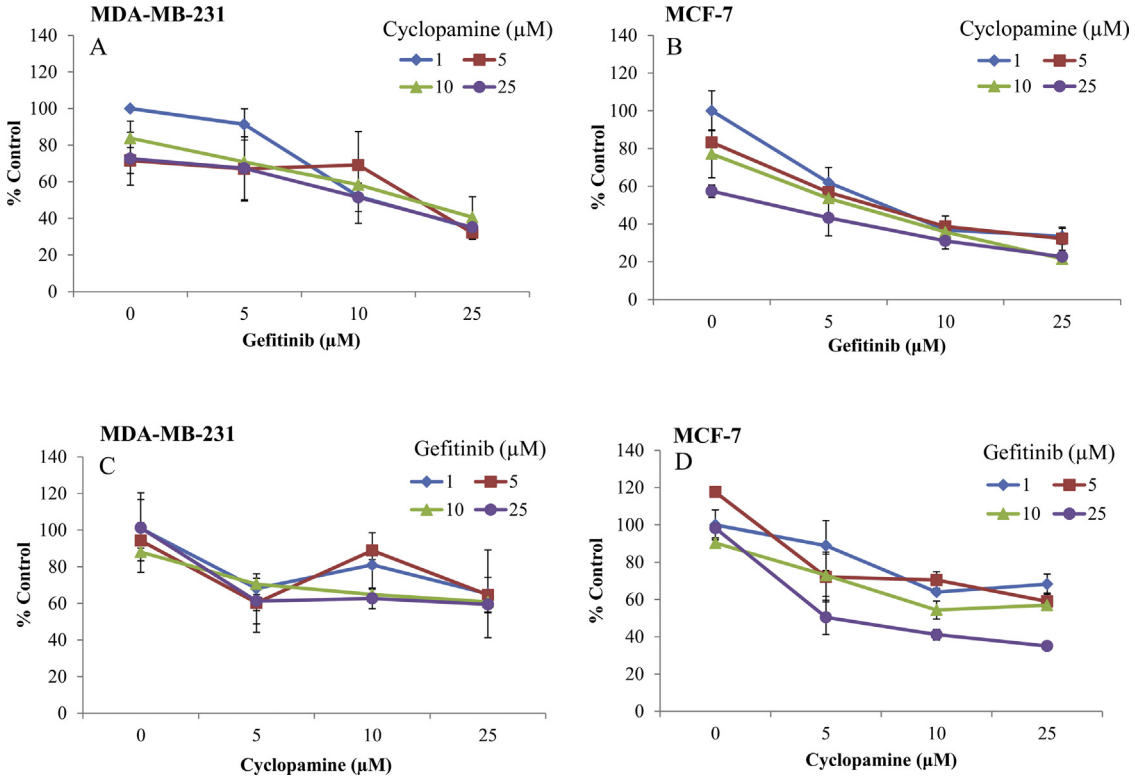

Fig. 6. Dose–effect curves for gefitinib and cyclopamine combination in human breast cancer cells. Sequential exposure (36 h incubation periods to gefitinib followed by cyclopamine in MDA-MB-231 and MCF-7 cells, respectively (A) and (B). Sequential exposure (36 h incubation periods) to cyclopamine followed by gefitinib in MDA-MB-231 and MCF-7 cells, respectively (C) and (D). Cell viability was determined using MTT assay and plotted as a percentage of control.

Table 3
Combination index (CI_{50}) analysis of simultaneous and sequential treatment of human breast cancer cell lines MDA-MB-231 and MCF-7 for 72 h using afatinib or gefitinib and cyclopamine. Molar ratio of afatinib or gefitinib to cyclopamine is 1:1.

Drug combination	Cell line	Time	CI_{50}	Interpretation
Afatinib → cyclopamine	MDA-MB-231	72	0.24	Synergism
Afatinib → cyclopamine	MCF-7	72	2.65	Antagonism
Cyclopamine → afatinib	MDA-MB-231	72	3.90	Antagonism
Cyclopamine → afatinib	MCF-7	72	2.52	Antagonism
Gefitinib → cyclopamine	MDA-MB-231	72	2.87	Antagonism
Gefitinib → cyclopamine	MCF-7	72	3.56	Antagonism
Cyclopamine → gefitinib	MDA-MB-231	72	5.20	Antagonism
Cyclopamine → gefitinib	MCF-7	72	3.12	Antagonism

The CI values are interpreted as follows: <1.0, synergism; 1.0 additive and >1.0, antagonism. Each experiment was done in triplicate.

approximately 44% of cells being apoptotic while gefitinib and cyclopamine combination caused apoptosis 28% of cells. Our findings suggest the level of apoptosis observed in the combination groups to be slightly higher than that obtained by adding the level of apoptosis detected in the corresponding monotherapy groups.

We also determined the effect of combining gefitinib and cyclopamine on the migration ability of MDA-MB-231 cells using the scratch wound assay. Under our conditions, gefitinib demonstrated better inhibition of cell migration compared to cyclopamine and the ability of gefitinib to inhibit cell migration appeared to be dose-dependent (Fig. 7C and D). Importantly, superior inhibition of cell migration was observed in the group treated with combination of gefitinib and cyclopamine compared to untreated control and each drug alone. Similar studies were performed for afatinib and cyclopamine, however, the treatment killed majority of cells following the 48 h exposure.

3.7. Gli-1-expression in MDA-MB-231 is downregulated by combination of cyclopamine and gefitinib

Gli-1 has been implicated in breast cancer cell proliferation. Therefore, we examined the effect of cyclopamine and gefitinib alone and in combination on Gli-1 expression in MDA-MB-231 cells. The cyclopamine (10 μM) treated group decreased Gli-1 expression by ~60%, which was 2 fold more than that observed in the gefitinib (10 μM) treated group. Nonetheless, combination of cyclopamine and gefitinib was more potent in downregulating Gli-1 expression compared to monotherapy and resulted in repressing Gli-1 expression by more than 80% (Fig. 7E).

4. Discussion

Majority of breast cancer patients relapse and progress to metastatic disease despite initial positive response to

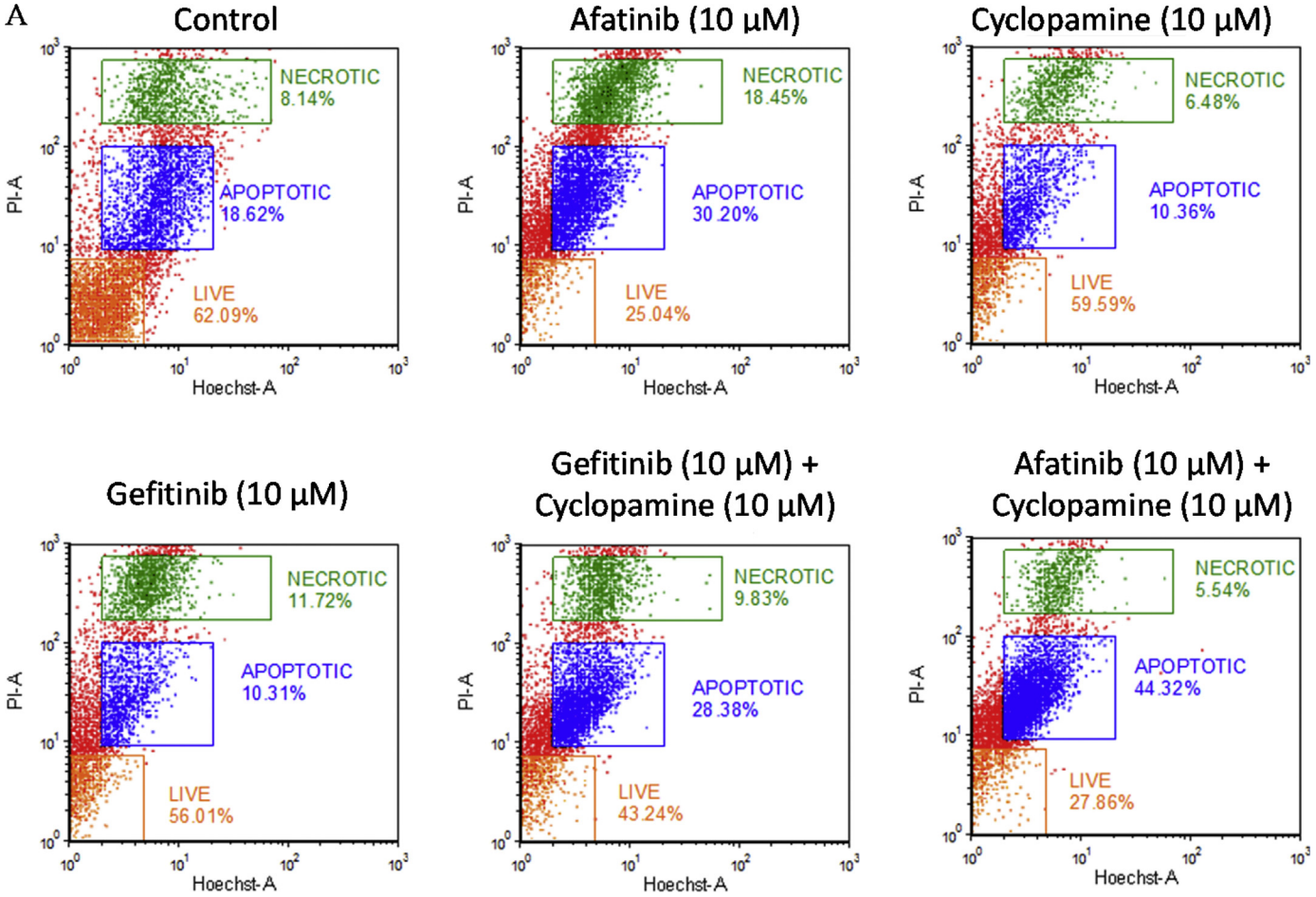

Fig. 7. Effect of afatinib, gefitinib and cyclopamine alone or in combination on apoptosis and cell migration in MDA-MB-231 human breast cancer cells. (A) Hoechst 33342/propidium iodinde (PI) staining was used to determine rate of apoptosis following treatment of cells. (B) Quantitative representation of apoptosis in cells following treatment. (C) Micrographs demonstrating effect of cyclopamine and gefitinib on breast cancer cell migration. MDA-MB-231 cells were grown to 70% confluence in six well plates and three parallel wounds made using pipette tip. Cells were treated with cyclopamine (10 or 20 μM) or gefitinib (10 or 20 μM) alone or in combination for 48 h after which cells were washed with ice cold 1× PBS and imaged under an inverted microscope. (E) Gli-1 expression in MDA-MB-231 breast cancer cells quantified by real time polymerase chain reaction following treatment with cyclopamine (10 μM) and gefitinib (10 μM) alone or in combination.

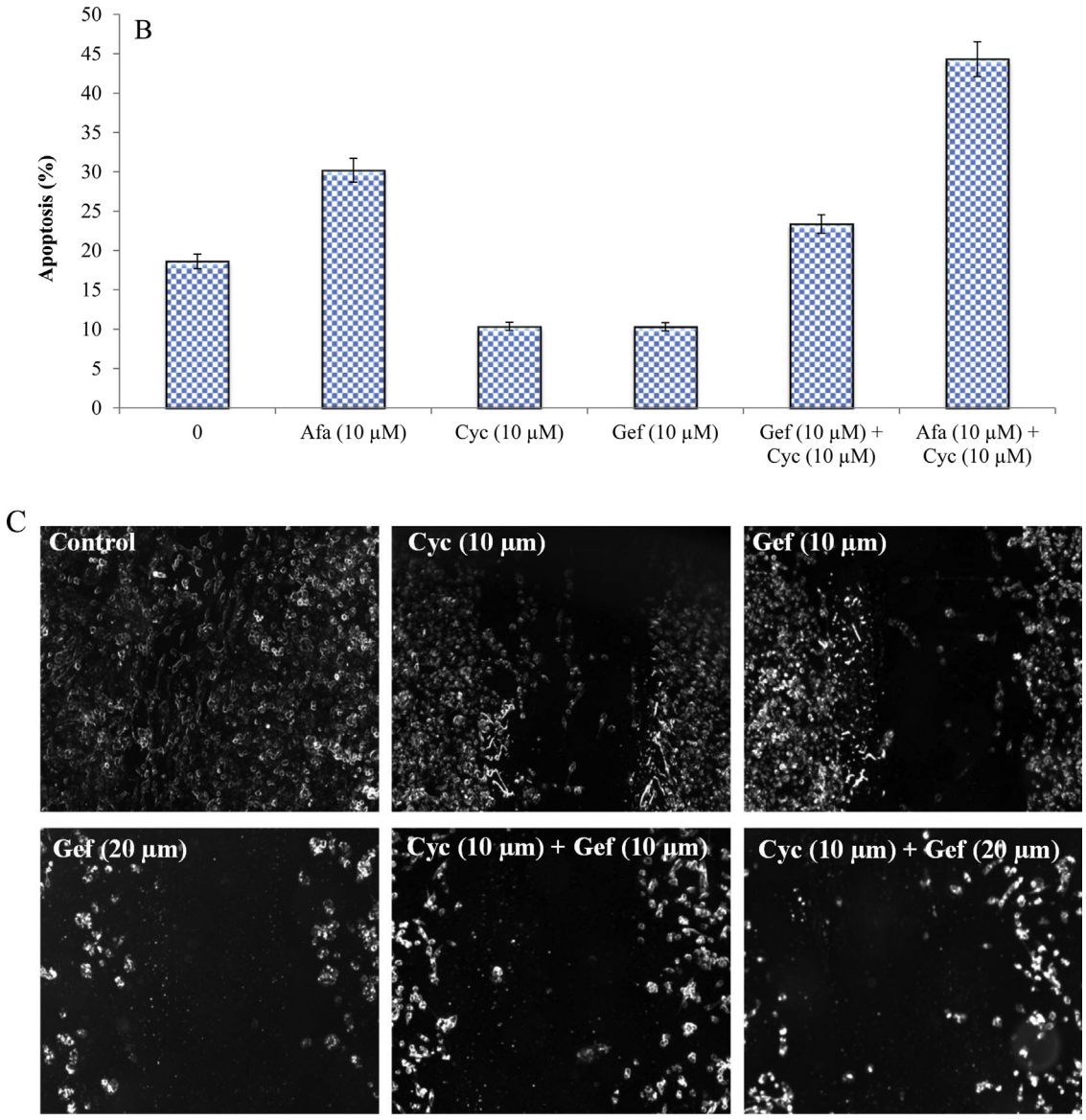

Fig. 7. (Continued)

chemotherapy. The reasons for chemoresistance include overexpression and dysregulation of EGFR and hedgehog signaling components. Hedgehog pathway regulates breast cancer stem cells and is implicated in tumor regeneration while EGFR is pivotal to survival, growth, invasion and metastasis. The principal aim of this study was to demonstrate the potential benefit of combining hedgehog inhibitor (cyclopamine or vismodegib) and EGFR inhibitor (afatinib or gefitinib) in providing an effective rational approach for treating localized and metastatic breast cancer tumors.

To correlate cyctotoxic effect of combination therapy with EGFR and hedgehog status, we first performed a comparative mRNA analysis of the endogenous expression of EGFR and hedgehog signaling elements (Gli-1, SHH and SMO) in MCF-7 and MDA-MB-231 breast cancer cells. These cell lines were chosen for the present study to reflect early stage (MCF-7) and advanced stage (MDA-MB-231) breast cancer. It is important to note that MCF-7 is estrogen receptor positive (ER^+), progesterone receptor positive (PR^+), human epidermal growth factor receptor 2 negative ($HER2^-$) and is typically endocrine and chemotherapy responsive [43]. In contrast, MDA-MB-231 is triple negative (ER^-, PR^-, $HER2^-$) and exhibits intermediate response to chemotherapy [43]. Our results reveal EGFR expression to be 40-fold higher in MDA-MB-231 compared to MCF-7 while Gli-1 and SHH expression was 1.5-fold and 4-fold higher in MCF-7 cells compared to MDA-MB-231, respectively (Fig. 1). Importantly, while MCF-7 expressed SMO, no SMO expression was detected in MDA-MB-231 cells. Our trends for Gli-1 and SHH expression are in good agreement with reported results of Sun and coworkers and Muhkerjee et al. [13,44]. However, Sun and coworkers observed at least a four-fold higher expression in MCF-7 compared to MDA-MB-231 for Gli-1 while Muhkerjee et al. reported at least a 100-fold higher expression in MCF-7 compared to MDA-MB-231 for SHH [13,44]. Interestingly, Muhkerjee et al. also detected SMO expression in both MCF-7 and MDA-MB-231 while we observed SMO expression only in MCF-7 cells [13]. Our findings confirms that overexpression of EGFR might be a characteristic factor associated with aggressive forms of breast cancer while upregulated expression of hedgehog signaling elements is more associated with early stage breast tumors.

Since, we were interested in identifying a potent hedgehog antagonist for this study, we evaluated the antiproliferative effects cyclopamine and vismodegib in MCF-7 and MDA-MB-231 breast

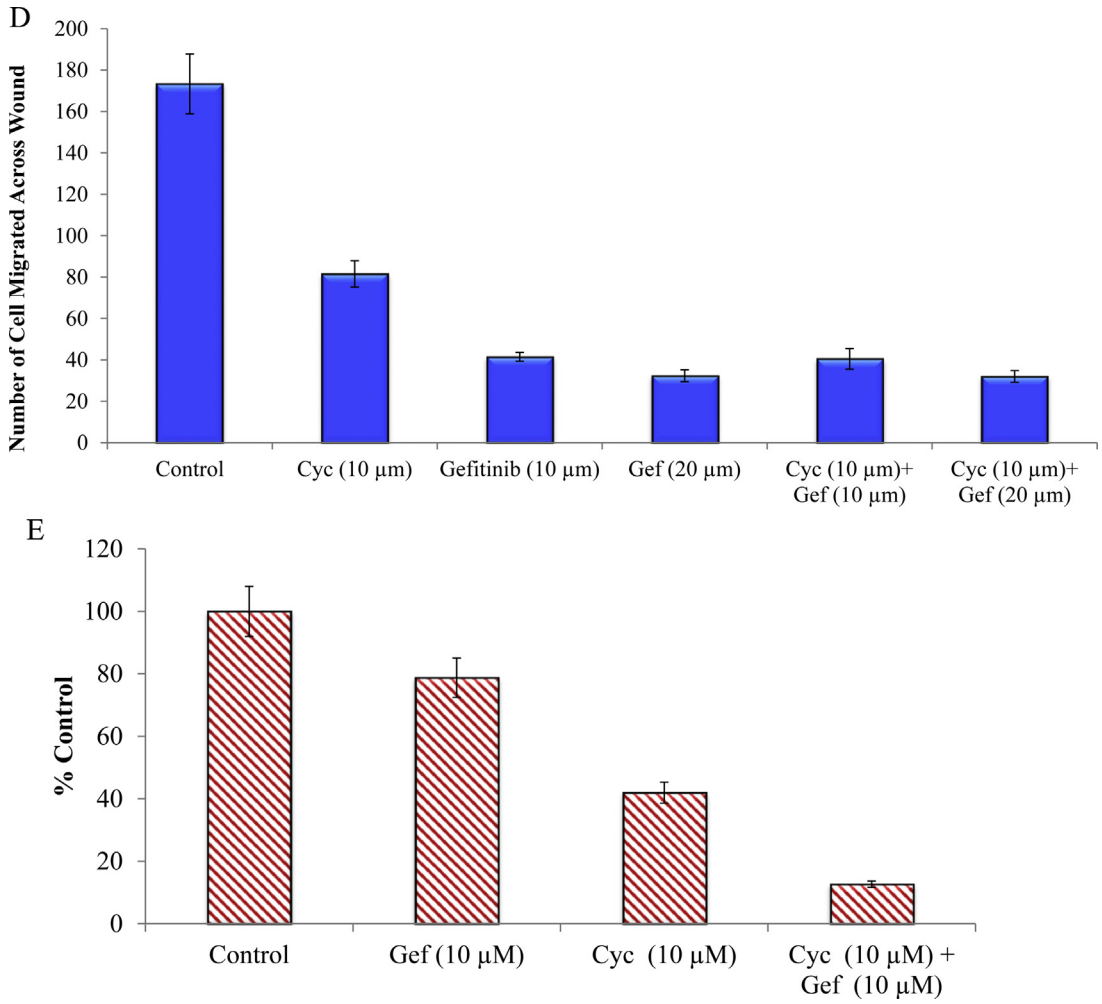

Fig. 7. (Continued)

cancer cells. Regardless of time, both cyclopamine and vismodegib exhibited dramatically stronger inhibitory effect in MCF-7 cells compared to MDA-MB-231 (Fig. 2 and Table 1). We postulated that this observation may be due to higher endogenous expression of hedgehog signaling elements in MCF-7 compared to MDA-MB-231. Since both cyclopamine and vismodegib are SMO antagonists, we attributed the comparatively superior antiproliferative effects observed in MCF-7 to the presence of SMO, especially since no SMO expression was detected in MDA-MB-231 cells in our study. It is noteworthy, that cyclopamine was more potent in inhibiting cell growth compared to vismodegib regardless of cell line or exposure time. However, it is not clear why this is the case since vismodegib is a second generation hedgehog antagonist and expected to be a better SMO antagonist. The relative therapeutic benefits of afatinib and gefitinib were also ascertained by evaluating the biological effects of both drugs on cell proliferation in MCF-7 and MDA-MB-231. Our data show both drugs to be more potent in MDA-MB-231 cells compared to MCF-7 cells. Also, afatinib more potently inhibited cell growth compared to gefitinib regardless of cell line. In both instance, it appears the superior antiproliferative effects are due to the relatively higher endogenous expression of EGFR in MDA-MB-231 cells and the fact that afatinib effectively inhibits kinase activity of both wild type and EGFR, HER 2 and ErbB4 mutations [45,46].

We examined simultaneous combination of cyclopamine and afatinib and cyclopamine and gefitinib on MCF-7 and MDA-MB-231 breast cancer cells. Response surface methodology analysis suggests synergistic interaction between cyclopamine and afatinib at lower concentrations and at shorter exposure time irrespective of cell lines (Fig. 3). It appears that at longer exposure times afatinib kills majority of cells at the concentrations examined and hence the potential for synergistic interaction between afatinib and cyclopamine is lost since it is overshadowed by the individual potency of afatinib. In the case of cyclopamine and gefitinib combinations, synergistic interactions occur at lower concentrations in MCF-7 cells regardless of exposure time. On the other hand, combination of cyclopamine and gefitinib was synergistic over a wider concentration range in MDA-MB-231 cells particularly at longer exposure times (Fig. 4). Cyclopamine is more potent in MCF-7 cells with IC_{50} values at least half those observed in MDA-MB-231 cells at corresponding time points. In contrast gefitinib is more potent in MDA-MB-231 cells with the lowest IC_{50} observed at the longest exposure time studied (72 h). Consequently, we hypothesized that the extent of synergistic interaction between cyclopamine and afatinib or cyclopamine and gefitinib in our study correlates with EGFR expression and therefore the ratio of the concentration of EGFR inhibitor to concentration of hedgehog inhibitor.

To elucidate this assertion, we also examined the type of interaction occurring for combination of cyclopamine and afatinib and cyclopamine and gefitinib in MCF-7 and MDA-MB-231 cells when molar ratio of afatinib or gefitnib to cyclopamine is 5:1 (Table 2). Using combination index analysis our data reveal strong synergistic interaction between cyclopamine and afatinib in both

cell lines and time points except for MCF-7 at 24 h. In contrast, combination of gefitinib and cyclopamine was synergistic in MDA-MB-231 cells and antagonistic in MCF-7 cells notwithstanding exposure times. It is important to note that synergism is more rigorously defined in the combination index method compared to the response surface method or clinical synergism. One chief conclusion of this study is that the therapeutic effect observed for combination of cyclopamine and afatinib or cyclopamine and gefitinib is comparatively better in MDA-MB-231 cells than in MCF-7 cells. This may be partially attributed to the endogenous expression levels of EGFR and hedgehog signaling elements in both cell lines. As mentioned previously, endogenous expression of hedgehog signaling elements is modestly higher in MCF-7 cells compared to MDA-MB-231 cells while endogenous expression of EGFR is significantly higher in MDA-MB-231 cells compared to MCF-7 cells. We presume a "concurrent blockade of multiple pathways" hypothesis to be the mechanism governing our combination therapy [47]. Hence, observed therapeutic effects may depend on the trade-off between EGFR inhibitor versus hedgehog inhibitor efficacy. It is likely that the effects of EGFR inhibitors predominates that of the hedgehog inhibitor in our study. Our findings therefore appear to confirm the notion that the presence and extent of synergistic interactions is more dependent on the EGFR inhibitor than the hedgehog inhibitor. Exposure schedule can affect therapeutic outcomes of combination therapy. Hence, we examined the antiproliferative effect of sequential treatment on MCF-7 and MDA-MB-231 cells where cells were exposed to each drug (10 μM) for 36 h (Fig. 5 and Table 3). Our results regarding sequential combination treatment are rather interesting. Although several studies have demonstrated sequential administration of chemotherapy followed by EGFR inhibitors to improve therapeutic outcome, we did not observe this trend in our study [48–51]. In fact, strong antagonism was seen when breast cancer cells were exposed to cyclopamine first followed by and EGFR inhibitor. Among the treatment schedules studied, only afatinib followed by cyclopamine in MDA-MB-231 cells produced notable synergistic interaction. Although, it is not clear why afatinib followed by cyclopamine results in synergistic effects, our result do not appear to be an anomaly since they were performed in triplicate.

We further probed into how hedgehog and EGFR signaling pathways might cooperate to mitigate apoptosis and enhance cell migration. Indeed, our results showed that combination of cyclopamine and afatinib and cyclopamine and gefitinib more potently induced apoptosis and inhibited cell migration in MDA-MB-231 cells compared to monotherapy (Figs. 6 and 7). These findings are in accordance with the studies of Mimeault et al. which reported induction of a massive rate of apoptotic death in prostate cancer cells following treatment with cyclopamine in combination with gefitinib [38]. To clarify the potential interaction between EGFR and hedgehog signaling pathways, we examined Gli-1 mRNA expression levels following treatment with gefitinib and cyclopamine alone or in combination. We found cyclopamine alone or in combination with gefitnib dramatically decreased Gli-1 expression in MDA-MB-231 cells which is in good agreement with the literature [39,52].

Collectively, our findings reveal that combination of cyclopamine and afatinib or gefitinib synergistically inhibits proliferation of MDA-MB-231 and MCF-7 breast cancer cells. However, the potential and extent for synergistic interaction was found to be dose, time, cell line and schedule dependent. Also, combination therapy more effectively induced apoptosis, inhibited cell migration and downregulated Gli-1 expression compared to monotherapy in the breast cancer cells studied. Based on the present study, combination therapy simultaneously targeting EGFR and hedgehog pathways can be potentially beneficial in treating breast cancer. Nonetheless, further examination of the underlying cellular mechanism governing the observed synergistic effects needs to be conducted using a more expansive set of breast cancer cells consisting of a wide variety of EGFR status. These studies also need to be confirmed in vivo to assess potential for clinical translation. Additional understanding obtained from these investigations should furnish beneficial data for EGFR and hedgehog based combination therapy for treating breast cancer and other solid tumor cancers.

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

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