

Modulatory effect of sodium butyrate on anticancer activity of abemaciclib in MDA-MB- 231 human breast cancer cells

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Abstract

Triple negative is the most aggressive subtype of breast cancer with its treatment limited only to chemotherapy and radiotherapy. The effect of abemaciclib and /or sodium butyrate on MDA-MB-231 triple negative breast cancer cells was investigated. Using MTT assay, IC₅₀ for the growth inhibitory effect of abemaciclib, sodium butyrate, and their combination were 14.55 μM, 7.08 mM, 3.743 mM, respectively. The combination showed a synergistic interaction with a decrease in IC₅₀ to 2.55 μM of abemaciclib and 3.74 mM of butyrate. Three replica of four groups of cancer cells were treated for 48hr with IC₅₀ of either abemaciclib or butyrate or their high or low doses combinations. A fifth group was treated with complete medium and served as control. Cell migration, protein expression level of cyclinD1, E2F2 transcription factor, phosphorylated AKT, nuclear factor kappa B (NF-κB), cyclin dependent kinase-2 (CDK2), retinoblastoma (Rb), p16^{INK4a}, p53 and mRNA level of CDK2, p16^{INK4a} and p53 were assessed in all treated groups. Combination treatment of abemaciclib and butyrate showed a significant attenuation of cell metastasis. The combination treatment was associated with a decrease in E2F2, CDK2, and NF-κB protein level together with an attenuation in AKT phosphorylation level. The combination also showed an elevation in Rb, p16^{INK4a}, together with a reversal of DNA hypo-methylated state. Although abemaciclib monotherapy failed to alter CD1 or P53 levels, the combination significantly reduced CD1 level together with an increase in P53 level. In conclusion, combining butyrate with abemaciclib augmented its antiproliferative and antimetastatic effect and induced apoptotic activity.

Introduction

Breast cancer is the second leading cause of death after lung cancer in the world (Rakha et al., 2010). One in each 8 women has the risk of experiencing breast cancer throughout her life time (Kamińska et al., 2015). Breast cancer is classified according to histopathology of the cells, grade, stage and the molecular profile of the tumor.(Rakha et al., 2010) According to the molecular profile, breast cancer is classified into four types; luminal A (estrogen receptor positive (ER+), progesterone receptor positive (PR+) and human epidermal growth factor receptor negative breast cancer (HER2-)), luminal B (ER+, PR + and HER2+), HER2 enriched (ER-, PR- and HER2+), and triple negative breast cancer (TNBC) which lacks the expression of ER, PR, and HER2 (Perou et al., 2000).

TNBC is one of the most aggressive subtypes of breast cancer which is distinguished by high proliferation rate and high ability to metastasis to distant organs such as the brain and lung, in comparison with other types which metastasis to the bone and soft tissues (Uscanga-Perales et al., 2016). Due to the aggressiveness and lack of ER, PR and HER2 expression in this type of cancer, chemotherapy remains the only therapeutic option.

In the last decade several common hallmarks of cancer progression have been identified which participate in transformation of normal cells into malignant cells. Among those hallmarks, is genetic mutation or epigenetic alteration, uncontrolled cell proliferation, and resistance of apoptosis (Hanahan and Weinberg, 2011). Epigenetic control of gene expression is defined as mitotically heritable change in

gene expression without changing in DNA sequencing (Jovanovic et al., 2010) via small tags on histone protein or DNA sequence (Sharma et al., 2010). In human cells the building unit of the chromosomes is the nucleosome, which consists of 147 base pair of DNA wrapped around octamer histone (H2a, H2b, H3, and H4), and exterior H1 histone protein which keep DNA wrap in place. The structure of chromatin is controlled by acetylation and methylation of the lysine residue of the histone tails (Sharma et al., 2010). Post translational modifications of histones are controlled by histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs) (Black et al., 2012). The balance between those enzymes maintains the chromatin structure in an accurate state and that leading to accurate gene expression according to cell needs and function (Sharma et al., 2010). In certain type of TNBC, there is imbalance between those enzymes, such in mesenchymal stem like breast cancer cells which show an overexpression of HDACs (Kalle and Wang, 2019).

HDACs are classified into 4 classes according to their structure, class I (HDAC 1,2,3,8), class IIa (HDAC 4,5,7,9) /class IIb (HDAC 6,10), class III (SIRT1-7), class IV (HDAC 11). These enzymes have the ability to remove acetyl group from histone proteins thereby affecting gene expression, and also removing acetyl group from expressed non-histone protein thus altering their biological activity (Seto and Yoshida, 2014).

DNA methylation is the second most common epigenetic modification; methylation of cytosine-guanine (CpG) islands at the gene promoter leads to silencing of gene expression, in contrast methylation on the gene body that leads to activation of gene expression. Alteration in DNA methylation in cancer is common, as most cancer types show a global hypomethylation at CpG islands with specific hypermethylation at the promoter of the tumor suppressor genes leading to genetic instability and silencing of tumor suppressors (Kondo, 2009) .

As epigenetic alteration has a remarkable contribution in tumor formation, so most of recent researches are directed to epigenetic drugs as anti-tumor therapy (Temian et al., 2018). One of the most common epigenetic drugs are HDAC inhibitors, such as sodium butyrate (NaBu) (Garpis et al., 2017). NaBu is a short chain fatty acid which is produced naturally in the colon due to bacterial fermentation of fibers, and it acts as an inhibitor of HDACs class I/IIa/IV (Damaskos et al., 2017). The anti-tumor activity of NaBu was reported in previous studies against various types of cancer including breast cancer (J. Chen et al., 2019; W. Huang et al., 2019).

Cell cycle process is controlled by cyclins and cyclin dependent kinases, which are defined as cell cycle drivers. Cell cycle is also governed by cyclin dependent kinase inhibitors which are the cell cycle brakes. Uncontrolled cell proliferation is caused by alteration in cell cycle regulators protein and gene expression. (Vermeulen et al., 2003). In the last decade, synthetic drugs which target cell cycle pathway had been developed such as palbociclib, ribociclib, and abemaciclib. These drugs are approved by FDA for treating metastatic hormonal positive breast cancer, as they improved patient outcomes and significantly increased survival rate (O'Brien et al., 2018).

Abemaciclib, is ATP competitive inhibitor of CDK4/6 with a higher selectivity for CDK4 over CDK6. This reduces the degree of neutropenia usually associated with inhibition of CDK6, thus avoiding the need for

an interruption in dosing as with other CDK4/6 inhibitors. Abemaciclib was approved on February 2018 by FDA for the treatment of advanced metastatic hormonal positive breast cancer, as a monotherapy or in combination with endocrine therapy (O'Brien et al., 2018). However, despite its success in breast cancer treatment, some patients showed acquired or de-novo resistance. Several mechanisms were found to be related to the development of abemaciclib resistance.

The present study investigated the possible modulatory effect of NaBu on the anti-tumor activity of abemaciclib and the effect of NaBu on some of the multiple mechanisms that contribute to abemaciclib resistance.

Material And Methods

Cell culture

Human MDA-MB-231 triple negative breast cancer cells were purchased from American Type culture collection (ATCC), conducted in center of excellence for research in regenerative medicine and its applications (CERRMA). Breast cancer cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin under a humidified atmosphere containing 5% CO₂ at 37°C. The cells (70% confluent) were treated with 14.5µM abemaciclib LY2835219 (Selleckchem Co-USA) and/or 7 mM NaBu (Sigma Co-USA) and incubated in CO₂ incubator for 48 h.

Cell viability assay

To investigate the growth inhibitory effect of abemaciclib and NaBu as single drugs or in combination, MDA-MB-231 cells were transferred to 96-well plate at a density of 7000 cells/well and incubated at 37°C for 24 h. The cells were then treated with different concentrations of abemaciclib (0.09-11.58 µM) and/or different concentrations of NaBu (1.325-16.96 mM) and incubated for additional 48 h. The effect of all single and combinatorial treatments on cell viability was assessed using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (van Meerloo et al., 2011). Briefly, after incubation period, MTT reagent was added to each well at a final concentration of 0.5 mg/ml. The plate was incubated at 37°C for 4 h in CO₂ incubator. 100µL of dimethyl sulfoxide (DMSO) was added to solubilize formazan crystals. Then the plate was shaken gently in darkness for 15 min. The optical density (OD) was measured at 590 nm using ELISA microplate reader. Relative viability of each treatment was analyzed based on the absorbance of each treated sample compared to the control sample.

The IC₅₀ of each single drug and their combination were determined using Compusyn software. To determine whether the interaction was synergistic or antagonistic, the combination index (CI) was determined as described by Chou (Chou, 2010), where CI<1 indicates synergism, =1 indicates additive action, and >1 indicate antagonism. The dose reduction index (DRI), which signifies how many folds of dose reduction for each drug at a given effect are allowed in synergistic combination, was then determined.

Five experimental groups were used. The first group was treated with complete medium and served as a control group. The four remaining groups were treated with either abemaciclib or NaBu or their combinations for 48hr. Three replicas of each group were treated with the IC₅₀ of each single drug or their combination for 48hr. The nuclear extract or cells lysate was processed by RT-PCR or ELISA, respectively to quantify the mRNA level and protein level of the investigated parameters.

Quantitative real-time PCR

Total RNA was extracted using miRNeasy mini kit (#217004, QIAGEN). Then, total mRNA was reverse transcribed to complement DNA (cDNA) and quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Rotor-Gene SYBR Green Kit (#204174, QIAGEN) according to the manufacturer's instructions. Quantitative RT-PCR was performed using Rotor-Gene cyler Q-pure detection system version 2.1.0 (QIAGEN, USA). The primers used in qRT-PCR analysis were as follows: human TP53 (5'-CCAGCCAAAGAAGAAACCA -3' Forward, 5'-TATGGCGGGAGGTAGACTGA-3' Reverse); human CDKN2A (5'-CCCAACGCACCGAATAGTTA-3' Forward, 5'-GCATGGTTACTGCCTCTGGT-3' Reverse); human CDK2 (5'-GTCCCCAGAGTCCGAAAGAT-3' Forward, 5'-GCTTTCTGCCATTCTCATCG-3' Reverse). Human β -Actin was chosen as endogenous control (housekeeping genes). For one step RT-PCR, the reaction mixture (25 μ l) contained 1 μ l template RNA, 12.5 μ l 2x Rotor-gene SYBR Green RT-PCR Master Mix, 2.5 μ l of each set of oligonucleotide primers, 0.25 μ l Rotor-Gene RT Mix, and 6.25 μ l RNase-free water. The one step real-time PCR conditions included a reverse transcription at 55°C for 10 min, an initial denaturation at 95°C for 5 min, followed by a 40-cycle amplification consisting of denaturation at 95°C for 5 sec, and combined annealing/extension at 60°C for 10 sec followed by melting curves to verify qRT-PCR product identity. All reactions were examined in triplicate for each sample. All primer pairs were checked using primer blast. Relative mRNA expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Quantification of protein expression level of cell cycle regulatory proteins in control and treated cells was measured using ELISA Kits. Proteins detected were: human cyclinD1 (ab214571 SimpleStep, Abcam), human PRb using human PRb (MBS2508443, Mybiosources), E2F transcription factor 2 (E2F2) (#abx151366, Abbexa,UK), human CDK2 using human CDK2 (#MBS2510990, Mybiosources), CDKN2A (p16) (ab227903 SimpleStep, Abcam), p53 using (ab171571-p53 human SimpleStep, Abcam), phosphorylated AKT (pS473) (#KHO0111, Invitrogen), NF- κ B (#MBS450580, Mybiosources). Global DNA methylation (5-methylcytosine) was determined using ab233486 colorimetric kit (Abcam). All kits were used according to manufacturer's protocol..

Wound healing assay

MDA-MB-231 cells were seeded into 6 well plates and left to grow for 48 h. Confluent monolayers were gently scratched using a sterile 1ml pipette tip across the center of the well. Cells were washed twice with complete media to remove detached cells, then cells were replenished with 2 ml new complete media supplemented with 10% FBS and 1% Pen-Strep, afterward, abemaciclib and/or NaBu were added to the

wells. Images were captured for each well at zero time, and after incubation for 24 h and 48 h using inverted microscope. The percent cell migration was expressed as the number of migrated treated cells relative to control.

Statistical analysis

All results were presented as mean \pm S.D. Computer software GraphPad Prism version 9.00 for Windows, GraphPad Software, USA, was used for all statistical analyses and graphical data presentations. For the comparison between experimental groups, one-way analysis of variance (ANOVA) (non-parametric) was used, followed by Tukeys's multiple comparisons test, Differences with values of $P < 0.05$ were considered statistically significant.

Results

Cytotoxicity assay

The growth inhibitory effect of abemaciclib and NaBu (alone or in combination) on MDA-MB-231 was determined using MTT assay. Cell viability of MDA-MB-231 cells was reduced after abemaciclib and/or NaBu treatment for 48 h in a concentration dependent manner. (Fig. 1) IC_{50} of abemaciclib, NaBu, and their combination were 14.55 μ M, 7.08 mM, 3.743 mM, respectively. Microscopical examination confirmed the higher potency of the combination on cell viability (Fig. 2). The combination of abemaciclib and NaBu showed a synergistic interaction as the calculated CI value was found to be less than 1 (CI = 0.70399). The calculated DRI of both drugs at 50% cell viability were 5.69 and 1.89 for abemaciclib and NaBu respectively and it was translated into a decrease in IC_{50} to be equal to 2.55 μ M of abemaciclib and 3.74 mM of NaBu. Three replicas of four groups of cancer cells were treated for 48hr with IC_{50} of either abemaciclib or butyrate or their combinations. Two combinations were used; high dose combination (combination 1) and low dose combination according to the dose reduction index (combination DRI). A fifth group with treated with complete medium and served as control as shown in Table (1).

Table 1
Experimental groups, and the IC_{50} of each drug in treated groups

Experimental group	IC_{50} of abemaciclib	IC_{50} of NaBu
Control	-	-
Abemaciclib	14.55 μ M	-
NaBu	-	7.08 mM
Combination1	14.55 μ M	7.08 mM
Combination (DRI)	2.55 μ M	3.74 mM

Expression of cell cycle drivers; cyclinD1, E2F2, and CDK2

Abemaciclib as a single treatment caused a remarkable decrease in E2F2 protein level and CDK2 gene and protein level in comparison with untreated cells, without showing any significant change in the protein expression level of cyclinD1. On the other hand, NaBu as a mono therapy showed a significant decrease in cyclinD1, E2F2 protein level, and CDK2 gene and protein level. Combinations treatment showed a significant decrease in cyclinD1 protein level in comparison with abemaciclib as a single drug (Fig. 3)

AKT phosphorylation level and NF- κ B protein expression level.

Phosphorylation level of AKT at S473 and the NF- κ B protein level were determined in all experimental groups. As shown in figure (4) abemaciclib as a single drug caused a remarkable decrease in both AKT phosphorylation level at S473 and NF- κ B protein expression level in comparison with untreated cells. On the other hand, NaBu as a single drug significantly reduced NF- κ B protein expression level and it had no effect on AKT phosphorylation level at S473 in comparison with untreated cells (control). The combinations treatment did not show any further decrease neither in AKT phosphorylation level nor in NF- κ B protein expression level compared with abemaciclib as a single drug

Expression level of tumor suppressors; PRb, p16 and p53.

The protein level of the hypophosphorylated form of retinoblastoma (Rb) and the gene and protein expression level of p16 and wild type p53 tumor suppressors were measured. As shown in figure (5), abemaciclib caused a significant elevation in PRB protein level, p16 gene expression and protein level, but its up-regulating effect on p53 was restricted to gene expression only without any significant elevation in protein level, in comparison with the untreated cells. On the other hand, NaBu showed a significant elevation in PRb protein level, p53 gene expression and p53 protein level, but it had no effect on p16 gene or protein expression level. The combinations treatment showed a further elevation in PRB protein expression level in comparison with each single drug. While combination 1 produced a significant elevation in p16 gene and protein expression level and p53 protein level as compared with abemaciclib or NaBu, however p53 gene expression level was elevated in comparison with abemaciclib only. As compared with abemaciclib, combination 2 significantly elevated p53 protein expression level without significantly altering P53 gene level

5-methyl cytosine (5-mC) global DNA methylation in MDA-MB-231 cells.

To investigate the epigenetic effect of abemaciclib and NaBu (alone or in combination) on MDA-MB-231 cells, the 5-mC global DNA methylation of all treated and untreated cells was assessed. As shown in figure (6) abemaciclib and NaBu as single drugs did not affect the global DNA methylation in comparison with untreated cells, whereas combination 1 treatment caused global DNA hypermethylation in comparison with untreated cells or abemaciclib as a single drug.

Wound Healing Assay

To investigate the effect of abemaciclib and NaBu (alone or in combination) on cell migration in MDA-MB-231, the percent reduction in cell migration was assessed by the wound healing assay. As shown in figure (7), after 24hr or 48hr of treatment with each single drug alone or in combination the cell migration was significantly reduced in comparison with the untreated cells. Compared to abemaciclib, NaBu and combinations treatment were able to significantly reduce cell migration after 48hr. The inhibitory effect of combination 2 (DRI) on cell migration was significantly higher than combination 1 after 48hr.

Discussion

Disturbances in HDACs expression is implicated as a possible carcinogenesis trigger, due to its vital role in controlling the expression of many oncogenes and tumor suppressor genes as well as its involvement in controlling the activity of several transcriptional factors. (Seto and Yoshida, 2014). Uncontrolled cell proliferation is one of the hallmarks of cancer and one of its triggers might be through nuclear and cytoplasmic disturbances in HDAC expression (Sharma et al., 2010).

That's why Epigenetic modulators such as HDACs inhibitors have been tested in many studies as potential anti-cancer drugs. Indeed to date, the FDA has approved three HDAC inhibitors; vorinostat, romidepsin, and belinostat for cutaneous/peripheral T-cell lymphoma treatment (Mottamal et al., 2015). Sodium butyrate (NaBu), a short chain fatty acids which acts as class I/IIa HDAC inhibitor, is under clinical trials as anti-cancer agent against various types of cancer (Davie, 2003). The reported alteration in HDACs expression in MDA-MB-231 (Kalle and Wang, 2019) prompted us to investigate the possible modulatory effect of NaBu on abemaciclib anti-cancer activity. Abemaciclib is FDA approved CDK4/6 inhibitor and it is used for advanced metastatic hormonal positive breast cancer alone or in combination with endocrine therapy (O'Brien et al., 2018).

In the present study, MDA-MB-231 cells were found to show an overexpression of cyclin D1, and upon treating the cells with abemaciclib for 48h, it failed to reduce cyclin D1 protein level. This result is consistent with a previous study where abemaciclib failed to decrease cyclin D1 expression in colorectal cancer. (Lee et al., 2020) Abemaciclib also showed an elevation in the hypophosphorylated form of Rb tumor suppressor protein in the present study. Consistently, it was reported that abemaciclib treatment caused accumulation of cyclin D1 together with a partial inhibition in Rb phosphorylation in HCT116 colorectal cancer cell line (S. H. Chen et al., 2018). It is well recognized that abemaciclib through its inhibitory effect on CDK4/6 results in inhibition of Rb phosphorylation which restricts the cell cycle progression. (O'Brien et al., 2018). To confirm the implication of cyclin D1 accumulation in the partial inhibition of Rb phosphorylation, cyclinD1 knockout cells were treated with abemaciclib, and a complete inhibition in Rb phosphorylation was noted in colorectal cancer cells. (S. H. Chen et al., 2018)

In the present study, NaBu was able to reduce cyclin D1 protein level as well as elevate the hypophosphorylated form of Rb. This is consistent with the previously reported decrease in cyclin D1

level upon treatment of monoplastic leukemia cell line (SKM-1) and non-small lung cancer cell line (NCI-H23) with NaBu. (M. Huang et al., 2004; Pellizzaro et al., 2001) The ability of NaBu to decrease cyclinD1 expression might be due to its inhibitory effect on NF- κ B, which is considered as a direct transcription activator of cyclinD1. (Hu and Colburn, 2005) NaBu could also inhibit cyclin D1 expression through inhibition of the cytoplasmic HDAC6. (Kalle and Wang, 2019) Briefly, inhibition of HDAC6, leads to hyperacetylation and proteosomal degradation of HSP90 which in turn leads to unstabilization and degradation of EGFR, thus attenuating all mitogenic signaling pathways downstream of EGFR activation, such as MAPK/ERK1-2 and PI3K/AKT/mTOR signaling pathways (Wee and Wang, 2017). Inhibition of MAPK/ERK1-2 pathway attenuates the formation of AP-1 transcription factor which is a direct activator of cyclinD1 expression. In addition, inhibition of PI3K/AKT/mTOR signaling pathway activates GSK-3 β and that leads to phosphorylation and proteosomal degradation of cyclinD1 protein (Ghoneum and Said, 2019; Guo et al., 2011; Wee and Wang, 2017).

Various possible mechanisms could be linked to the effect of NaBu on Rb phosphorylation. First, NaBu is able to induce p53 wild type expression (J. Chen et al., 2019), this was confirmed in the present study, as NaBu elevated both the gene and protein level of p53. As p53 is a stress detector transcription factor, so its expression is elevated during DNA damage and nutrient deficient or any sort of cellular stresses such as that induced by drugs or free radicals. The expression of p53 controls the cell fate, as one of its roles is to directly induce the transcription of p21 and p27, which are endogenous CDKs/cyclins complexes inhibitors, with a consequent attenuation of Rb phosphorylation level, to stop the cell cycle progression and give much more time to the cell to repair the DNA damage or to restore its energy in case of nutrient deficient (Vermeulen et al., 2003). Thus the ability of NaBu to induce p53 expression could be linked to its ability to induce a cell cycle arrest as was noted in HCT116 colon cancer treated with NaBu (J. Chen et al., 2006).

It is worth mentioning that NaBu could activate p21 and p27 transcription through inhibition of PI3K/AKT signaling pathway secondary to inhibition of HDAC6 as mentioned before (Ghoneum and Said, 2019; Wee and Wang, 2017). Another possible mechanism for diminished Rb phosphorylation by NaBu treatment could be mediated through inhibition of nuclear HDAC1 which is an inhibitor of SP-1 transcription factor. Activation of SP-1 could also induces the transcription of p21. (Pajak et al., 2007) In addition, NaBu was reported to decrease c-myc oncogene expression which acts as a transcription repressor of p21. (J. Chen et al., 2019; La Monica et al., 2020)

In the present study the combination of NaBu and abemaciclib enhanced the ability of abemaciclib to induce a cell cycle arrest through decreasing cyclinD1 expression with a further elevation in hypophosphorylated Rb level and the effect was maintained in small doses combination.

Abemaciclib and NaBu as single drugs were able to reduce expression of the cell cycle driver E2F2 in the current study. This decrease could be mediated through their ability to reduce c-myc expression which is a direct activator of E2F2 expression. (Beier et al., 2000) Previously, a significant decline in c-myc level upon treatment of mutant non-small lung cancer cells with abemaciclib or NaBu treatment was noted (La

Monica et al., 2020). It was also reported that the degradation of HSP90 induced by NaBu causes unstabilization and degradation of E2F1, E2F2 and E2F3 (Kotwal et al., 2021).

Abemaciclib and NaBu as a single drugs attenuated CDK2 gene and protein level. Consist with this finding, it was reported that abemaciclib reduced CDK2 expression in a various cancer cells and it was considered as a pharmacodynamic biomarker, indicating the responsiveness of the patient to the treatment (O'Brien et al., 2018). A significant decrease in CDK2 expression upon NaBu treatment was also previously noted in monoplastic leukemia cells (M. Huang et al., 2004). This decrease could be due to the observed decrease in NF- κ B level which acts as a direct transcription activator of CDK2 gene, as well as indirect activator through increasing E2F3 level. (Ledoux and Perkins, 2014).

Recently, a link between CDK6 inhibition and NF- κ B phosphorylation, particularly P65 subunit was noted in cervical cancer cell line. CDK6 phosphorylates p65 subunit and ease its translocation to the nucleus leading to the transcriptions of genes implicated in cell proliferation, angiogenesis, metastasis and resisting apoptosis (Buss et al., 2012). Thus, inhibition of CDK6 by abemaciclib may lead to attenuation of NF- κ B transcription activity and its expression.

On the other hand, acetylation of p65 subunit could activate or repress p65 transcription activity according to the site of acetyl group. HDAC3 has the ability to remove the acetyl group from the inhibitory residues on p65 subunit. Thus inhibition of HDAC3 by NaBu could attenuate p65 transcription activity and in turns decrease NF- κ B level (Leus et al., 2016).

Abemaciclib reduced AKT phosphorylation level in the present study which goes in parallel with abemaciclib-induced decrease in non-small lung cancer cell line (La Monica et al., 2020) In contrast to other CDK4/6 inhibitors such as palbociclib which showed elevation in AKT phosphorylation level in MDA-MB-231 breast cancer cell line. On the other hand, NaBu had no effect on AKT phosphorylation as a single drug despite its ability to attenuate EGFR signaling pathway (W. Huang et al., 2019). A similar observation was noted in cervical cancer cell line (J. Chen et al., 2006).

By analyzing the cell cycle regulators, abemaciclib significantly elevated p53 gene expression but it failed to stabilize its protein level. Similarly, abemaciclib failed to induce p53 level in liposarcoma cell line (Sriraman et al., 2018). In various cancer types, abemaciclib failed to induce apoptosis and its effect was restricted to inhibition of cell proliferation (La Monica et al., 2020; Lee et al., 2020). On the other hand, NaBu showed a significant elevation in p53 gene and protein level. This finding is in line with the reported induction of intrinsic apoptosis, through p53 dependent pathways, in MCF7 breast cancer cell line and HCT116 colon cancer cell line following butyrate treatment (J. Chen et al., 2019). The mechanism by which NaBu stabilized p53 protein level might be through its inhibitory effect on HDAC1. Normally, HDAC1 deacetylates p53 protein leading to recruitment of MDM2 E3 ubiquitin ligase, and proteosomal degradation of p53. (Seto and Yoshida, 2014) Stabilization of p53 by NaBu allows it to binds to pro-apoptotic promoters and enhances their transcription (J. Chen et al., 2019).

It is worth mentioning that, the combination treatment showed a highly significant elevation in p53 gene more than each drug alone. It could be suggested that, despite the ability of abemaciclib to reduce AKT phosphorylation, and NF- κ B protein level which are correlated to inhibition of MDM2 activity and expression respectively, it failed to stabilize p53 protein. While upon adding NaBu in combination with abemaciclib, p53 stabilization was augmented through inhibition of MDM2 recruitment facilitated by inhibition of HDAC1 activity. This combination would be highly beneficial in MDA-MB-231 cancer cells as these cells are reported to show overexpression of both MDM2 (Gao et al., 2019) and HDAC1 (Park et al., 2011). Stabilization of p53 was observed even with low doses combination, thus the lower doses combination could be recommended to maintain the cytotoxic effect while preventing the large doses' side effects.

The effect of each single drug or the combinations on p16 was investigated. p16 is an endogenous CDK4/6 inhibitor which regulates cell cycle progression in normal cells as it act as cell cycle brake (Vermeulen et al., 2003). Cancer cells rely on attenuating those cell cycle brakes to ease the cell proliferation process during tumorigenesis (Vermeulen et al., 2003). It was reported that MDA-MB-231 shows a low expression level of p16 (de Oliveira et al., 2016). On the other hand, overexpression of p16 could be implicated in tumorigenesis, by inducing genomic instability which might lead to additional mutation as KRAS mutation. Overexpression of p16 may also lead to a decrease in cell dependence on CDK4/6 and overexpression of cyclin E and CDK2 to complete the cell cycle progression (Milde-Langosch et al., 2001). In addition, overexpressed p16 may translocate to the cytoplasm and enhance oncogenic biological functions (Milde-Langosch et al., 2001; Romagosa et al., 2011). Therefore, p16 overexpression is considered as one of the cell cycle specific mechanisms of resistance against CDK4/6 inhibitors (Pandey et al., 2019).

In the present study abemaciclib caused a significant elevation in p16 gene and protein expression level, On the other hand NaBu as a single drug had no effect on p16 expression level. Similarly, Pellizzaro, et al (2001) reported that NaBu caused no change in p16 expression level in NCI-H23 non-small cell lung cancer cell line (Pellizzaro et al., 2001) However, in colon cancer cells, butyrate caused an elevation in p16 expression. (Schwartz et al., 1998).

Combination treatment of NaBu and abemaciclib showed a significant increase in p16 gene and protein level in comparison with abemaciclib. This effect was evident only with the high dose combination. Thus using the low dose combination could be preferable as the high dose may enhance abemaciclib resistance through increase p16 level.

One of the characteristic features of TNBC, is its high ability to migrate to distant organs especially the brain and the lung and this contributes in making TNBC a lethal and aggressive breast cancer subtype. The molecular mechanism that underlie cell migration in TNBC is the low cadherin 3/4, and the high level of EMT biomarkers such as snail.. In the present study after 24h of treatment, the cell migration was significantly reduced in all treated groups with no differences between the single drugs and the combination treatment. (Ocana andPandiella, 2017). After 48h, NaBu and the combinations treatment

showed a further decrease in cell migration in comparison with abemaciclib. The anti-migration effect was more pronounced in low dose combination supporting the beneficial value of low dose combination over high dose combination. Anti-metastatic effect of abemaciclib and NaBu was previously noted in Caco-2 colorectal cancer cells (Lee et al., 2020) and MDA-MB-231 cells respectively (Park et al., 2011)

The anti-migration effect of both drugs may be linked to their ability to reduce NF- κ B and elevation of active Rb protein level. NF- κ B acts as a direct transcription activator of metalloproteinase 9 (MMP-9) (Wang et al., 2018). MMP-9 has the ability to degrade the extracellular matrix facilitating the migration of cells to the blood stream and/or lymph nodes (Wang et al., 2018). NF- κ B also stabilizes snail protein (Xia et al., 2014) which acts as a direct transcription repressor of cell adhesion molecule E-cadherin. Also, Rb protein act as a direct transcription activator of E-cadherin (Engel et al., 2015). Butyrate exerts an additional mechanism, as it attenuates AP-1 complex formation via inhibition of MAPK/ERK1-2 (W. Huang et al., 2019). AP-1 complex is implicated in metastasis as it is a transcription factor that induces the expression of MMP-9. (Wang et al., 2018)

In conclusion, as TNBC is the most aggressive breast cancer subtype so the need to develop a novel therapeutic protocol is of a great importance. Combining NaBu with abemaciclib could be a new therapeutic avenue as it enhanced the anti-tumor activity of abemaciclib through epigenetic regulation of cyclin D1, Rb, and p53 expression level. The combination also showed augmented anti-metastatic effect which was more pronounced with low dose treatment. This combination could improve treatment outcome and patient survival, as well as achieving the most challenging issue in cancer treatment that is reducing the dose without losing the drug effectiveness.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Esraa A. Abd ElAziz. The first draft of the manuscript was written by Esraa A. Abd ElAziz and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval :- Not applicable

Consent to participate:- Not applicable

Consent for publication :-Not applicable

Code availability :-Not applicable

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Figures

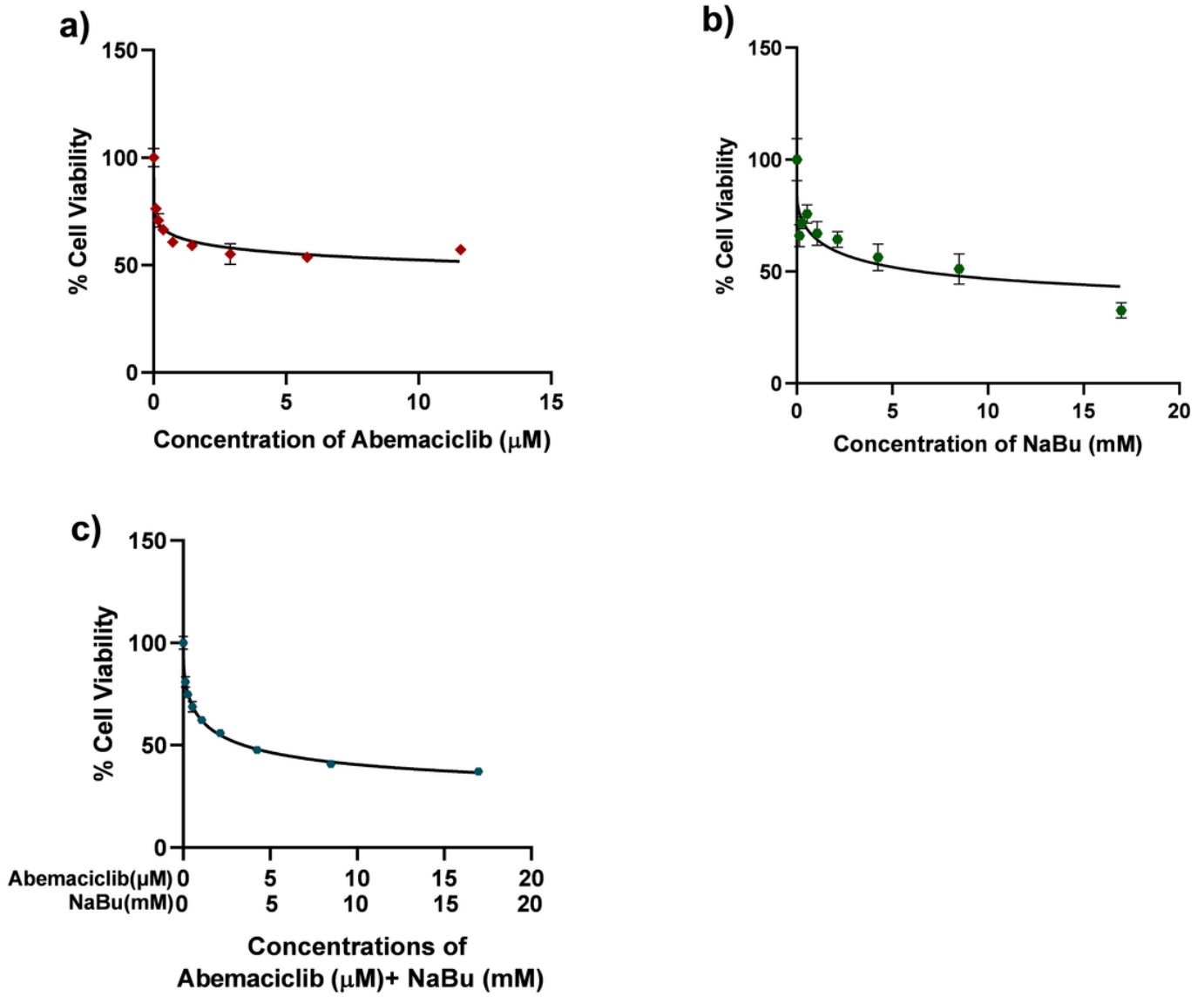


Figure 1

Dose response curve of MDA-MB-231 breast cancer cell line treated for 48hr with, a) Abemaciclib (0.09 -11.5808 μM), b) NaBu (0.1325 - 16.96 mM), c) Combination of abemaciclib and NaBu

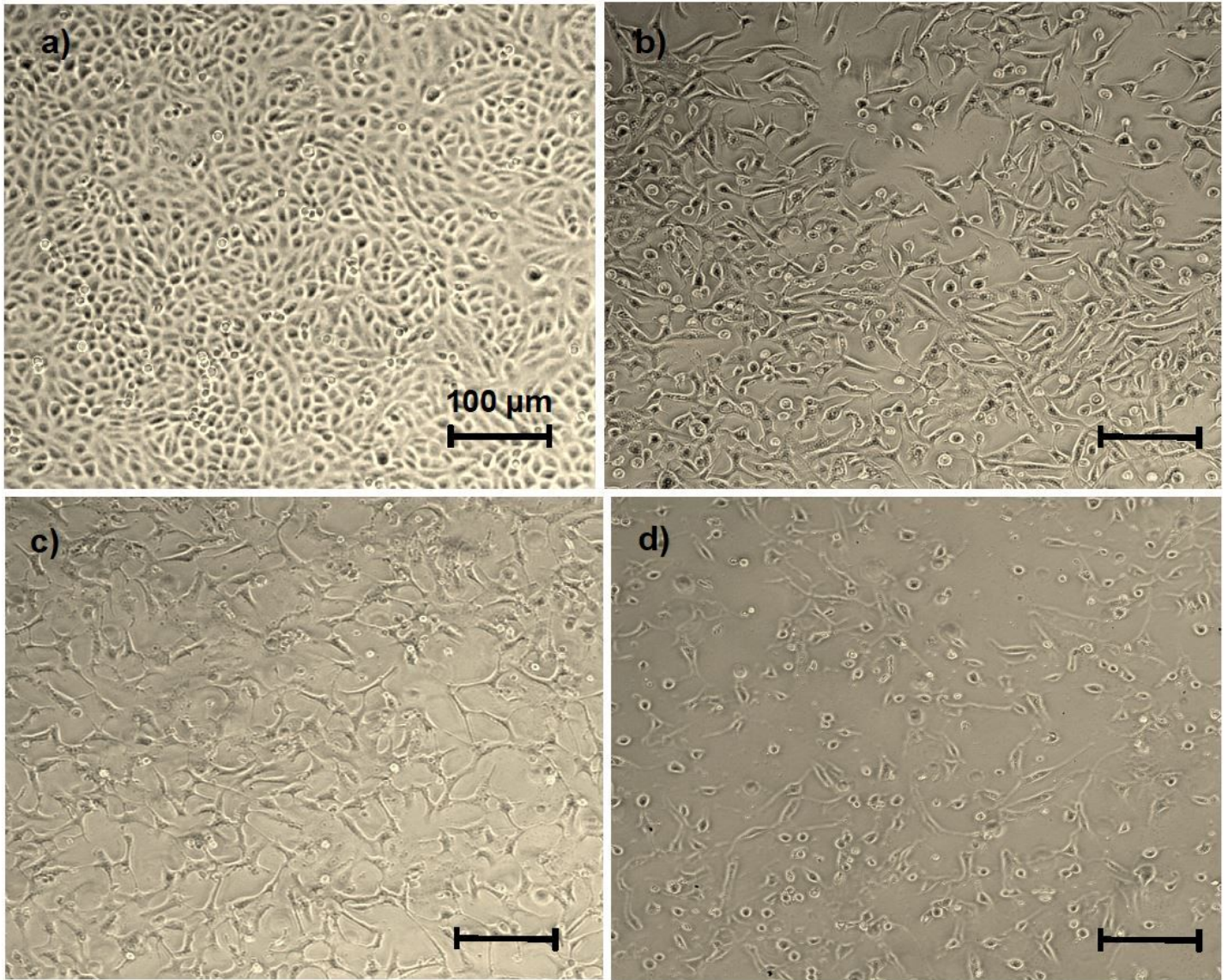


Figure 2

Microscopical representation of MDA-MB-231; a) untreated cells, b) treated with the 14.55 μM abemaciclib, c) treated with 7.08 mM NaBu, d) treated with the combination of 3.74 mM of NaBu and 2.55 μM abemaciclib, for 48hr

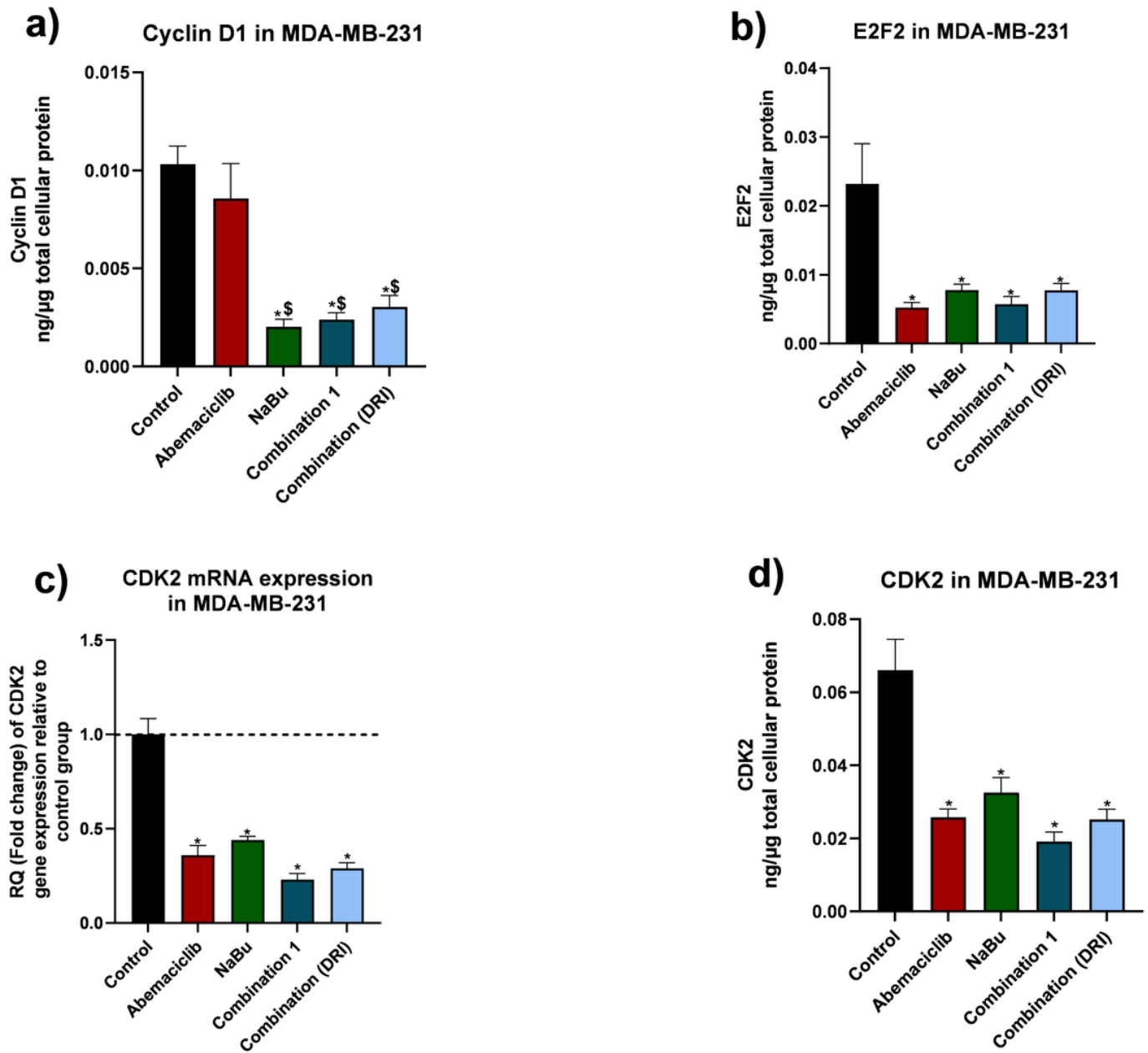


Figure 3

Effects of 48hr treatment of MDA-MB-231 human breast cancer cell line with abemaciclib, NaBu, or their combinations on; a) cyclinD1 protein expression level, b) E2F2 protein expression level, c) cyclin dependent kinase 2 (CDK2) mRNA gene expression level and d) CDK2 protein expression level. Data are expressed as the mean \pm SD (Standard deviation) of three samples each performed in triplicate. * P < 0.05 vs control, \$ P < 0.05 vs abemaciclib

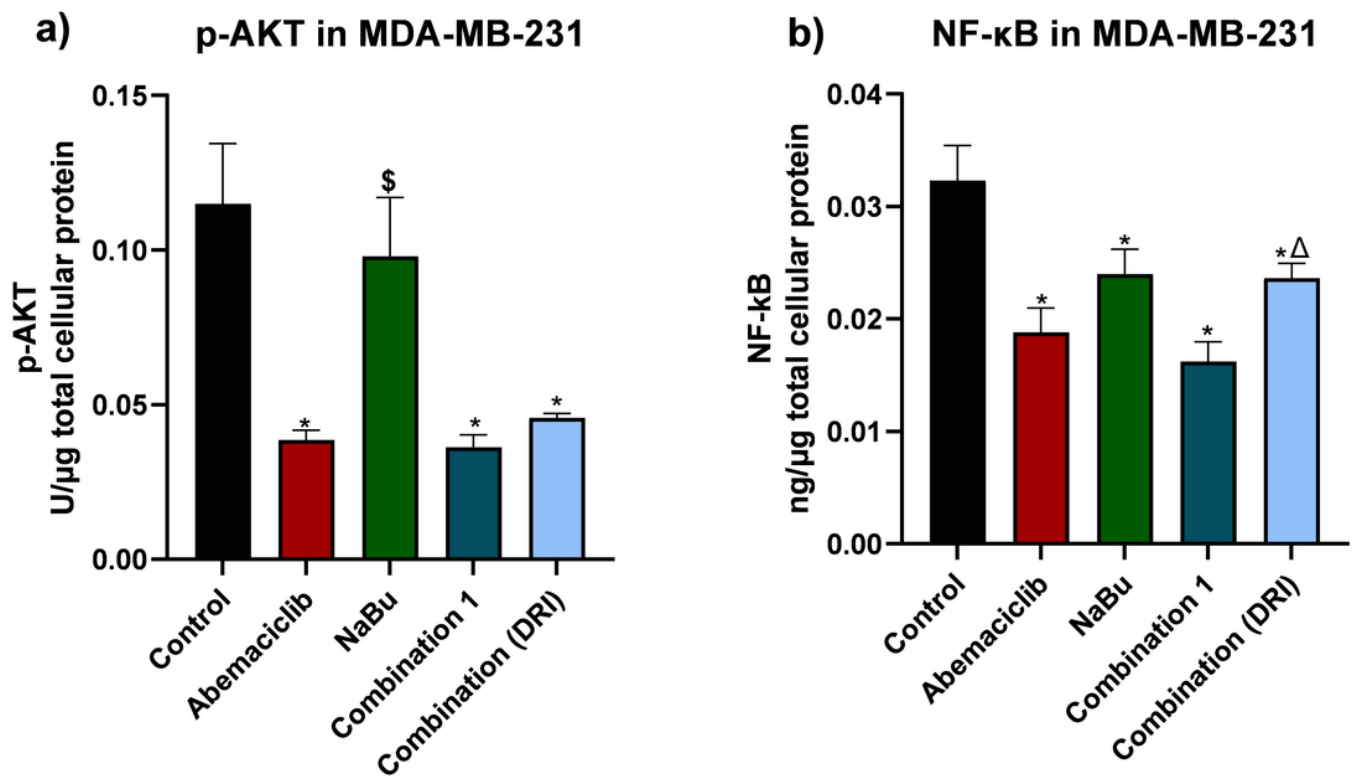


Figure 4

Effects of 48hr treatment of MDA- MB-231 human breast cancer cell line with abemaciclib, NaBu, or their combinations on; a) AKT phosphorylation level at S473, b) NF-κB protein expression level. Data are expressed as the mean \pm SD (Standard deviation) of three samples each performed in triplicate. *P< 0.05 vs control, \$ P< 0.05 vs abemaciclib, Δ P< 0.05 vs combination1

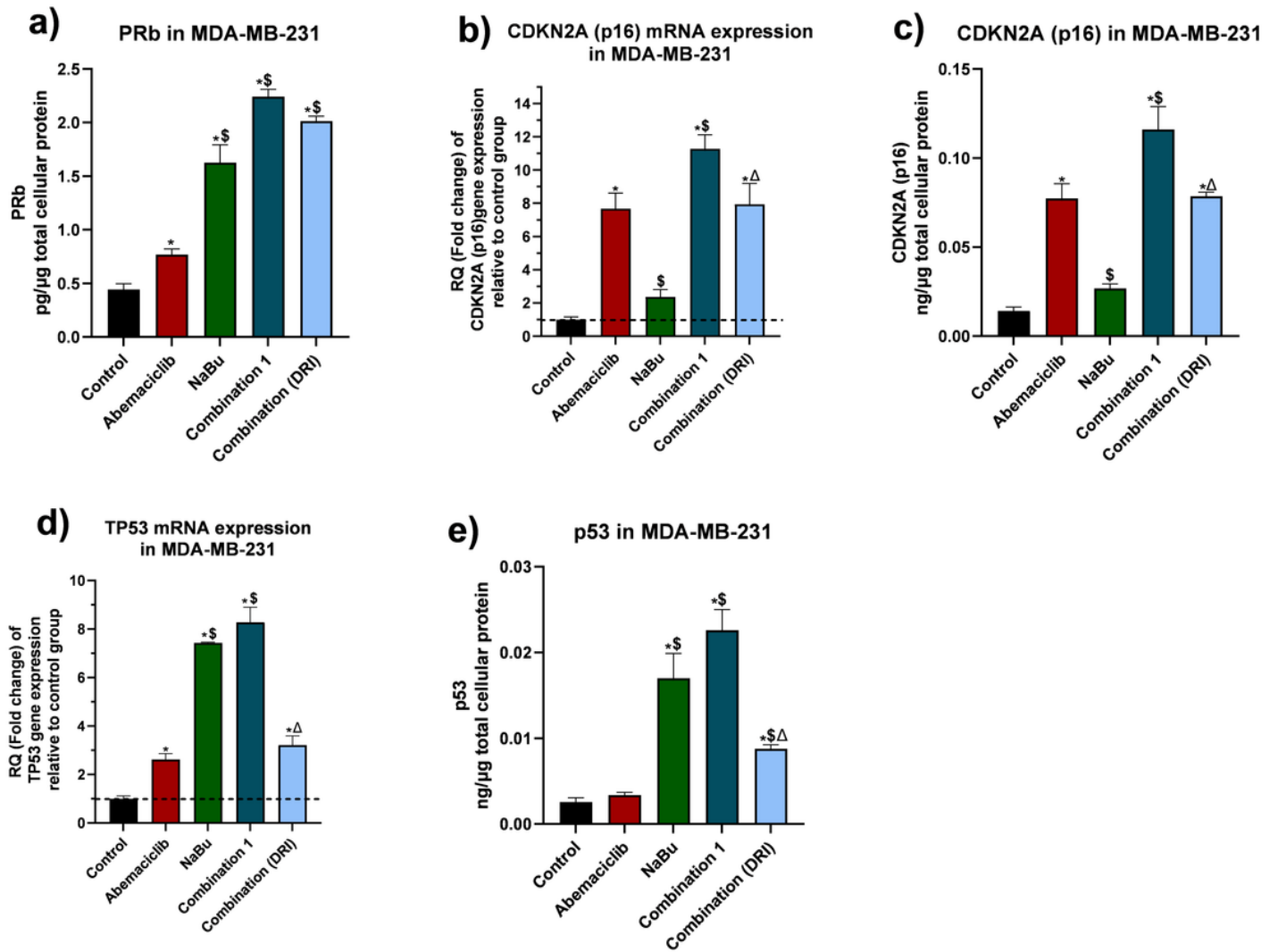


Figure 5

Effect of 48 hr treatment of MDA-MB-231 human breast cancer cell line with abemaciclib, NaBu, or their combinations on: a) PRb protein expression level, b) p16 gene expression level, c) p16 protein expression level, d) p53 gene expression level, e) p53 protein expression level. Data are expressed as the mean \pm SD of three samples each performed in triplicate. * $P < 0.05$ vs control, $\text{\$}$ $P < 0.05$ vs abemaciclib, Δ $P < 0.05$ vs combination1

Global DNA methylation (5-mC) in MDA-MB-231

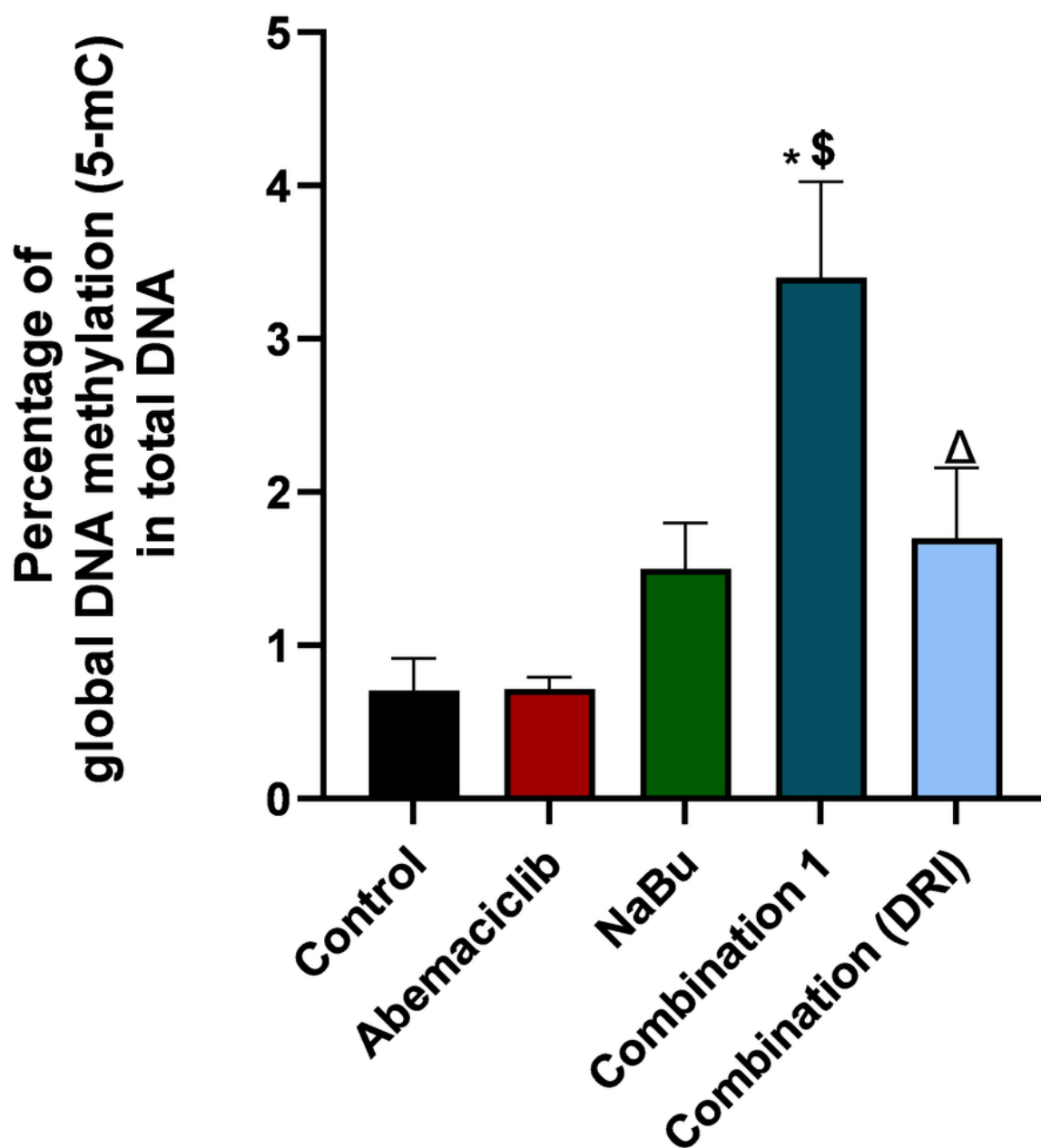


Figure 6

Effect of 48hr treatment of MDA-MB-231 human breast cancer cell line with abemaciclib, NaBu, or their combinations on global DNA methylation at 5-methyl cytosine. Data are expressed as the mean \pm SD of three samples each performed in triplicate. *P< 0.05 vs control, \$ P< 0.05 vs abemaciclib, Δ P< 0.05 vs combination1

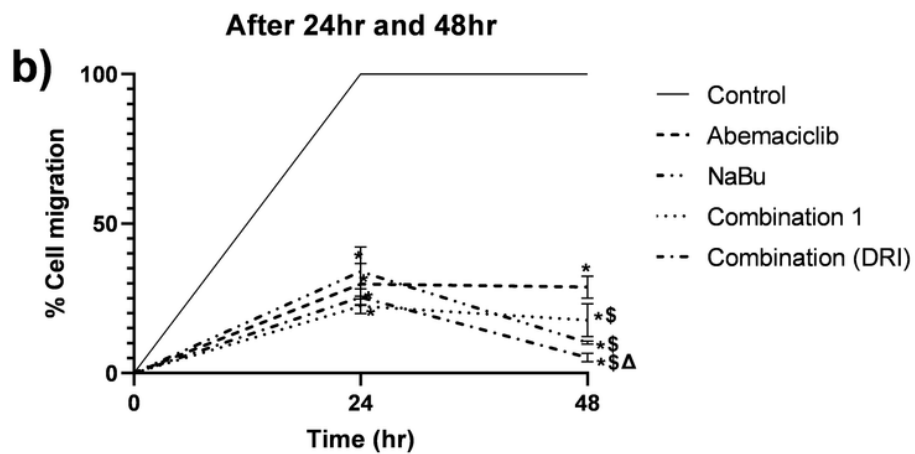
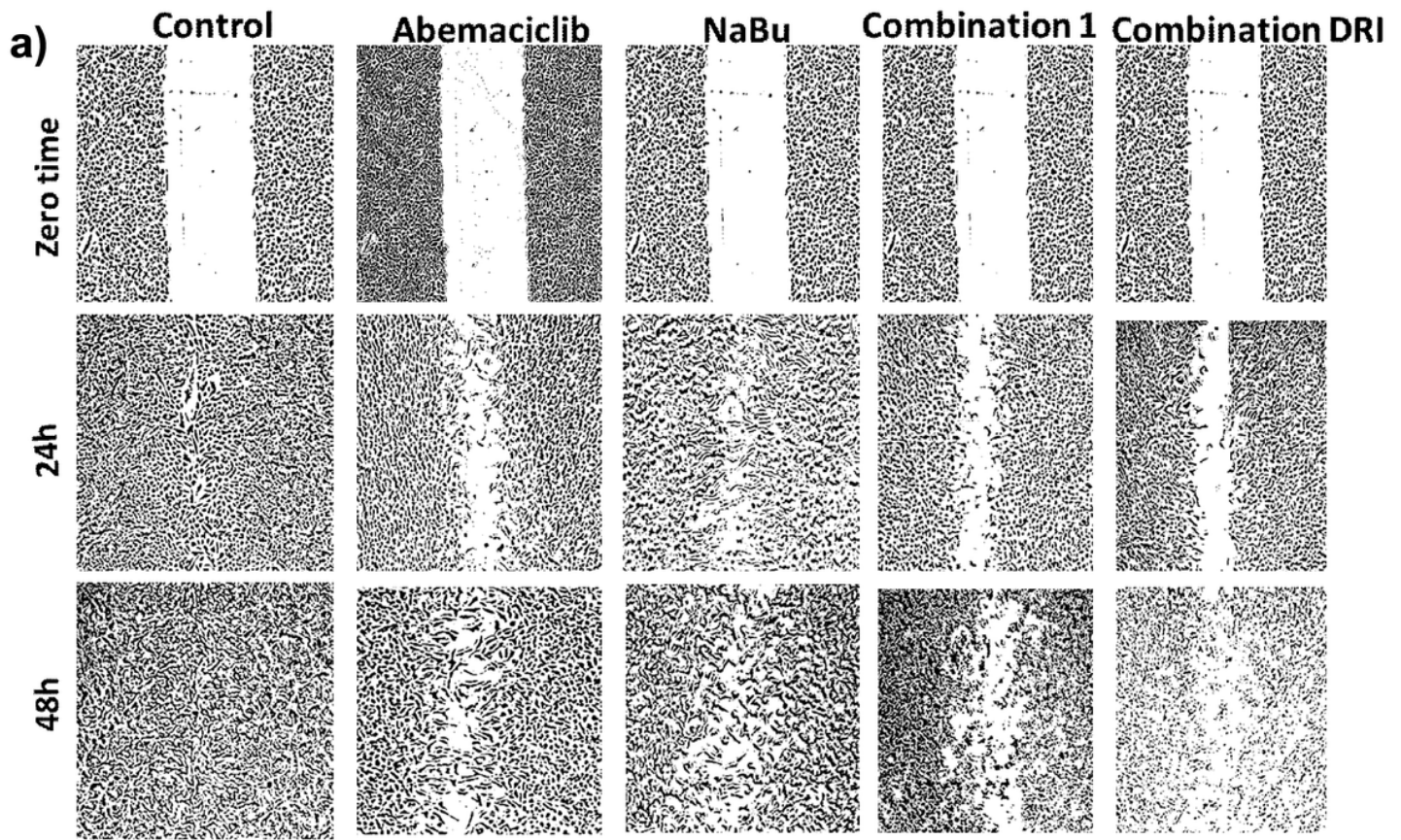


Figure 7

Effect of treatment of MDA-MB-231 human breast cancer cell line with abemaciclib, NaBu, or their combinations on cell migration. A) Microscopical examination of cell migration at zero time, 24hr and 48hr. B) line graph showing % cell migration at zero time and after 24hr and 48hr. Data are expressed as the mean \pm SD (Standard deviation) of three samples each performed in triplicate. *P<0.05 vs control, \$ P< 0.05 vs abemaciclib, Δ P< 0.05 vs combination1