

The Dicyano Compound Induces Autophagic or Apoptotic Cell Death Via Twist/C-Myc Axis Depending on Metastatic Characteristics of Breast Cancer Cells

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Abstract

Breast cancer is a heterogeneous disease which has distinct subtypes and therefore development of novel targeting treatments to fight against breast cancer is needed. Although autophagy and apoptosis considered as the major programmed cell death mechanisms are among the current target mechanisms, there are some difficulties in clinical treatment such as the development of drug resistance and cancer recurrence. Therefore it is important that illumination of distinctive mechanisms between cancer types for development novel treatment strategies. In this study, we examined the anti-proliferative effects of the triazole linked galactose substituted dicyano compound (hereafter referred to as the dicyano compound (the DC)) on two different breast cancer cell lines, MDA-MB-231 and MCF-7. We determined that response of each cell lines to the DC was different, since autophagy was induced in MDA-MB-231 and apoptosis was induced in MCF-7. For this reason, we hypothesized that these different responses may be due to the different characteristics of the cells and evaluated effects of aggressiveness degrees of both cell lines on response to the DC. As a result of our analysis, we determined that c-Myc regulation in both cell lines was different upon the DC treatment depending on expression of Twist, an epithelial-to-mesenchymal transition (EMT) mediator. Therefore, we suggest that Twist/c-Myc axis may have a role in different response to the DC-induced cell death pathways in breast cancer subtypes.

1. Introduction

Breast cancer is the second most common cancer type which is diagnosed approximately in one in eight women during their lifetime and is reason of 571.000 of 8.8 million cancer-related deaths (World Health Organization, 2015). Although breast cancer is the one of the most sensitive solid tumors to chemotherapy, drug resistance and tumor recurrence can develop in some of patients with breast cancer. Recurrence of breast cancer gives more metastatic characteristic to the cancer cells and promotes drug resistance. Unfortunately, drug resistance development is among the major problems in breast cancer fighting [1,2]. Also, all of breast cancer types cannot be treated same approach. For example, triple-negative breast cancer (TNBC) does not express estrogen and progesterone receptors and lacks human epidermal growth factor receptor 2 (HER2) overexpression or amplification. Therefore, hormonal therapies or treatments with related antibodies are ineffective for patients with TNBC [2,3]. In this context, development of novel anti-cancer agents, contributing to cancer treatment by targeting cellular mechanisms in cancer cells has a critical importance.

Various cellular events such as disfunctions of programmed cell death mechanisms and EMT contribute metastasis and recurrence of cancer [4,5]. While initial phase of metastasis progresses more rapidly, the percentage of cells that can pass to colonization stage, which is the last stage of metastasis, is only about 0.01%. As the reason of that invasive cell number decrease, it is considered as activations of programmed cell death mechanisms including autophagy and anoikis [6,7]. Autophagy is a process which is induced under stress conditions such as starvation and aims to maintain cell survival as long as possible. In this process, cytoplasmic content which includes organelles and proteins is degraded in lysosomal pathway. Recent studies have revealed that autophagy may function as a survival or a cell

death mechanism depending on type and stage of cancer. It is important to elucidate the details of this mechanism for autophagy-targeted cancer treatment [8]. Autophagy promotes cell growth and survival especially in solid tumor cells, since it performs energy and protein transduction. Therefore, blocking of autophagy may be a proper strategy for treatment of such tumors [9]. In contrast, it has been shown that autophagy depending on several and variable factors (cell type, signal type and duration, etc) could also cause tumor suppression, followed by the induction of apoptosis (Liang et al., 1999). Since autophagy could leads to tumor growth and tumor regression dependent on several and variable factors (cell type, signal type and duration, etc), all aspects of autophagy-induced cell death regared as a favorable strategy for chemotherapy should be further explained.

There are a number of studies showing that autophagy is related with apoptosis pathways [8,10–12]. Expression changes and mutual interactions of proteins associated with both autophagy and apoptosis pathways play crucial role to decide the cell fate concluding survival or cell death. The balance between apoptosis and autophagy can conclude in a different way. Autophagy may deplete inhibitors of apoptosis, so it can promote apoptosis [13,14]. Also, autophagy can play similar role with apoptosis for cell death by recruiting same signals. Alternatively, autophagy decreases cellular stress and delays initiation of apoptotic cell death. Moreover, apoptosis leads to cleavage of key proteins of autophagy (Atg proteins) and thus it can reduce autophagy and then fragments of these proteins can induce apoptosis [15–19]. Autophagy may provide elimination of cancer cells and it can be prefered when apoptotic pathway is blocked [20]. Autophagy can increase the effectiveness of cancer treatment either alone or in combination with other cell death pathways [21]. Several anti-cancer drugs that cause DNA damage and inhibit the polymerization of microtubule may also affect autophagy. However, the mechanism of action of these drugs on autophagy is not clearly known [22]. Although some studies have concluded opposite results, a number of studies suggest that inhibition of autophagy with cancer therapeutics reduces tumor cell death and promotes tumor growth [22–24]. The discovery of novel chemical agents targeting autophagy mechanism may be an alternative approach to development of anticancer-effective drugs.

Cancer cells could display variable characteristics depending on the origin and stage of the tumor (Blankenberg, 2009; Hoelder et al., 2012; Li et al., 2017). As is known, one of the main difference between cancer cells and normal cells is their energy metabolisms. Because cancer cells convertes the high amount of glucose to lactate even in the presence of oxygen (called as Warburg effect or aerobic glycolysis) (Liberti and Locasale, 2016). The novel chemical compound used in this study was synthesized by the scientists at The Department of Chemistry, Pamukkale University and its molecular structure was designed as compound (2,3-bis[1-(2,2,7,7-tetramethyltetra-hydro-bis[1,3]dioxolo[4,5-b;4',5'-d]pyran-5,methyl)-1H-[1,2,3]triazol-4-yl methylsulfanyl]-but 2-enedinitrile) to attach a sugar molecule at position of 6 [31]. Thus, cancer cells can recognize and metabolize this compound more and better than normal cells. In this study, we aimed to investigate the potential effects of the DC on programmed cell death mechanisms, especially autophagy in MDA-MB-231 and MCF-7 breast cancer cells, exhibiting metastatic and non-metastatic characteristics, respectively and found that Twist/c-Myc axis could be a decisive factor autophagy-induced apoptosis or autophagic cell death depending on metastatic

capacities of the breast cancer cells. Thus, our results indicates that the DC may have a potential as a therapeutic agent for breast cancer.

2. Materials And Methods

2.1 Cell Lines and Culture Conditions

MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 and MCF-7 cells were cultured in RPMI 1640 and DMEM culture medium, respectively, with 10% fetal bovine serum, 100 mg/mL penicillin, 50 mg/mL streptomycin and 1mM glutamine at 37°C in 5% CO₂.

2.2 Chemicals

2.2.1 Synthesis of 2,3-bis[1-(2,2,7,7-tetramethyl-tetrahydro-bis[1,3]dioxolo[4,5-b;4',5'-d]pyran-5,methyl)-1H-[1,2,3]triazol-4-ylmethylsulfanyl]-but-2-enedinitrile (the triazole linked galactose substituted dicyano compound (DC))

Preparation of the DC was explained in detail in our previous studies [31,32]. Molecular structure of the DC is shown in Fig. 1.

2.2.2 The activation and the inhibition of autophagy

We used chloroquine for blocking autophagy in the last stage of process. Chloroquine, the classic inhibitor of autophagy, accumulates in acidic lysosomes and increases lysosomal pH and hence inhibits lysosomal hydrolases and prevents autophagosomal fusion and degradation. In this study, we used 35 µM chloroquine for only MDA-MB-231 cell line during 24 hours in medium according to manufacturer's instructions (CST #14774). Torin1 is an effective inducer of autophagy and in this study, we used 100nM Torin1 for only MCF-7 cell line during 24 hours in medium according to manufacturer's instructions (CST #14379).

2.3 Cell Viability Assay by MTT

MDA-MB-231 and MCF-7 cells were seeded at a density 2.5×10^3 cell/well in 96-well plates and incubated during 24 hours in medium. After that, the cells were treated with the DC at determined concentrations (5,10,15, 20, 25 µM) or dimethyl sulfoxide (DMSO) during 48 hours. Cell viability was measured by using Vybrant® MTT Cell Proliferation Assay Kit according to the manufacturer's instructions (Thermo Fischer Scientific, Waltham, MA, USA). Formazan formation was measured spectrophotometrically at 560nm wavelength by Promega Glomax-Multi Microplate Reader.

2.4 Cell invasion assay

Invasion capability of the cells was analyzed by a Matrigel invasion chambers with 8-mm membrane pores (BD Biosciences, USA) as previously describe (Kucuksayan and Akca, 2017). Briefly, the cells cultured in serum-free conditions were seeded in upper chamber and the media containing 10% FBS was added to lower chamber. After that, the DC (1.46 μM) was added to both chambers to detect the effect of it on invasion capacity of MDA-MB-231. The cells were allowed to migrate for 22h at 37°C according to the manufacturer's instructions.

2.5 Western Blot Assay

All cell lysates were prepared in ice-cold RIPA lysis solution (CST #9806). Western blot analysis was performed as previously described [32]. Anti-LC3B (CST #14774), anti-BAX (CST #5023), anti-BCL-2 (CST #15071), anti-p53 (Santa Cruz, sc-55476), anti-p21 (Santa Cruz, sc-397), anti-cMyc (Santa Cruz, sc-40), anti-Twist (Santa Cruz, s-81417) and anti-GAPDH (Cell Signaling Technology #2118) antibodies were used.

2.6 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To assess apoptosis in breast cancer cell lines after treatment with the DC during 48 h, we used The CF® dye TUNEL Assay Apoptosis Detection Kit (Biotium Inc.), according to the manufacturer's instructions and as described previously [32].

2.7 Transient Transfection

Expression vectors pcDNA3.1-Twist and pcDNA3.1-Mock (for control) were used in this study. MDA-MB-231 and MCF-7 cells were seeded at a density of 2.5×10^5 cell/well in 6-well plates and were transfected with the plasmid vectors by using Lipofectamine 2000 (Thermo Fischer Scientific, USA) according to the manufacturer's instructions. 24 hours after transfection, the medium was discarded and cells were incubated in the medium with the dicyano compound for an additional 48h. Cell lysate preparation and western blot analysis were performed as described below.

2.8 Statistical Analysis

All in vitro studies were performed in triplicate and repeated independently to confirm the results. Statistical results of multiple experiments were shown as means \pm S.D. Student's t-test were performed for data analysis. Significance was determined with p-values of ≤ 0.05 .

3. Results

3.1 The DC has an anti-proliferative effect on MDA-MB-231 and MCF-7 cell lines as dose-dependent manner

We aimed to determine whether the DC has any effect on proliferation of MDA-MB-231 and MCF-7 cell lines. So, we incubated the both cell lines on determined concentrations of the DC (0.1 to 25 μM) during

48 hours. As seen in the Fig. 2, the DC had a significant anti-proliferative effect on the both cell lines. IC50 values of the DC were also determined on MDA-MB-231 (IC50 = 1.46 μ M) and MCF-7 (IC50 = 7.11 μ M). The results showed that MCF-7 cell line has been more resistant to the DC in comparison with MDA-MB-231 cell line. We predicted that non-metastatic characteristic of MCF-7 cell line may cause this resistance when compared with metastatic MDA-MB-231 cell line.

3.2 The DC induced autophagy depending on metastatic characteristics of breast cancer cells

In our previous study, we determined that the DC may induce autophagy in non-small cell lung cancer (NSCLC) cell lines [32]. Therefore, we firstly wanted to examine autophagy mechanism of breast cancer cells treated by the DC in this study.

Protein LC3B has two forms in cells: LC3BI (cytoplasmic (16kDa)) and LC3BII (on outer autophagosomal membrane (14kDa)). Expression of LC3BII is correlated with autophagosome formation and is used for determination of autophagy. So, we performed western blot assay for determination of autophagy mechanism in both breast cancer cell lines being treated with the DC in a time-dependent manner (0, 1, 2, 24, 48th hours). We used the DC in IC50 values for both cell lines. In MDA-MB-231 cell line, LC3BII expression was induced about 3-fold after 24-hour treatment by the DC and the induction of LC3BII expression was maintained even during 48-hour treatment (Fig. 3a). Although LC3BII expression was slightly induced in the first 2 hours, it was reduced dramatically after 48-hour treatment by the DC in MCF-7 cells (Fig. 3b). The results showed the DC induced autophagy in metastatic MDA-MB-231 cells while not induced in long-term manner in non-metastatic MCF-7 cells, suggesting that the DC-induced autophagy could depend on the metastatic characteristics of breast cancer cells.

3.3 The DC induced apoptosis only in non-metastatic breast cancer cells while it reduced invasiveness of metastatic one

As we thought that the DC leads to the induction of autophagy in MDA-MB-231 cells not in MCF-7, we performed flourometric TUNEL assay to determine whether it could induce or not apoptosis in both cell lines being treated with the DC during 48 hours and observed that the DC induced apoptosis in MCF-7, but not in MDA-MB-231. As we thought that the DC may cause induction of autophagy in MDA-MB-231, while it may cause induction of apoptosis in MCF-7, we assessed apoptotic cell death by TUNEL assay in both breast cancer cell lines which were treated with the DC during 48 hours. The results showed that the DC induced apoptosis in MCF-7, but not in MDA-MB-231 (Fig. 4a). To verify TUNEL results, we examined the expression levels of pro-apoptotic BAX and anti-apoptotic BCL-2 in the DC-treated cells by performing Western blot assay and determined about 1.5-fold increase in expression level of BAX in MCF-7 cell line while 1.5-fold decrease in MDA-MB-231 cell line (Fig. 4b). Conversely, Bcl-2 was decreased about 1.35-fold in MCF-7 cell line while it was increased about 1.7 folds in MDA-MB-231 cell line (Fig. 4c). So,

alterations in expression levels of BAX and BCL-2 in both cell lines are consistent with TUNEL assay results of each cell lines. The results revealed that the DC may cause two different types of programmed cell death in MDA-MB-231 and MCF-7 cell lines. Autophagic cell death occurs in MDA-MB-231 whereas MCF-7 cells undergo apoptotic cell death upon the DC treatment. Also, we wanted to evaluate effects of the DC on invasiveness of metastatic MDA-MB-231 cells. We treated the cells with IC50 value of the DC during 48 hours and observed reduction of invasiveness of the cells about rate of %50 as compared to untreated group (Fig. 5).

3.4 The DC regulates c-Myc and p21 in a p53-independent manner

After we established that non-metastatic breast cancer cells is more sensitive to the DC-induced apoptosis than metastatic one which autophagy is stably induced, we wanted to evaluate expression levels of crucial regulatory proteins (p21, p53 and c-Myc) implicated in cell cycle, DNA repair, apoptosis and survival. Thus, we observed whether there is alterations between expression profiles of these proteins in MDA-MB-231 and MCF-7 cells treated with the DC during 48 hours. Western blot assay revealed that p53 expression levels did not change significantly during 48-hour treatment in both cell lines. p21 expression were induced by 2.76 folds for 24-hour treatment and by 2 folds for 48-hour treatment in MCF-7 cells. Interestingly, the DC caused 1.52-fold increase of c-Myc expression in MDA-MB-231 cells whereas it dramatically repressed c-Myc expression in MCF-7 cells for 48-hour treatment (Fig. 6). Our results clearly showed that the opposite responses of breast cancer cell lines to the DC is due to c-Myc being regulated by p53-independent manner.

3.5 Autophagy is required to maintain the stabilization of c-Myc during the DC treatment

To identify whether autophagy induction by the DC directly correlates with c-Myc, we induced autophagy with Torin1 in MCF-7 cells preferring apoptosis instead of autophagy in response to the DC treatment. As consistent with our previous results, c-Myc expression was repressed by 2.56 folds by the DC in MCF-7 cells without Torin1. But, in the group with Torin1, c-Myc expression was repressed only by 1.2 folds by the DC in MCF-7 cell line. So, in autophagy induced conditions the DC-dependent c-Myc repression was lower in MCF-7 cell line (Fig. 7a). Meanwhile, the DC diminished c-Myc expression in MDA-MB-231 cells in presence of chloroquine, a strong inhibitor of autophagy (about 1.4 folds). Whereas, under normal conditions, c-Myc expression was induced by 1.3 folds upon the DC treatment (Fig. 7b). All these results revealed that the transcription factor c-Myc known especially as a cell cycle regulator could also be a marker to determine cell fate during autophagy.

3.6 Twist decides regulatory role of c-Myc for selection of cell death mechanism

Because of being a major transcriptional activator of Twist which plays crucial roles in metastasis especially EMT process and also has been shown to have a regulatory effect on autophagy [34–37], we

hypothesized that it could implicate in the DC-induced c-Myc downregulation in MCF-7 cells exhibiting an epithelial character. As we have confirmed by western blotting, endogenous Twist expression is lower in non-metastatic MCF-7 cell line than in metastatic MDA-MB-231 [38,39] (Fig. 8a). We transfected MCF-7 cells with pcDNA3.1-Twist vector to upregulate Twist and subsequently we verified Twist overexpression (Fig. 8b). Then, we treated the Twist-transfected MCF-7 cell line with the DC for 48 hours and observed that c-Myc was not downregulated upon the DC treatment in MCF-7 cells overexpressing Twist while c-Myc expression was decreased in presence of the DC treatment in the negative control MCF-7 cells (Fig. 8c). Consequently, we suggest that Twist/c-Myc axis may regulate the selection of cell death mechanism of breast cancer cells like a switch protein during the DC treatment.

4. Discussion

Breast cancer is a highly heterogeneous cancer type which possesses diverse phenotypic and morphologic features. Therefore, treatment strategies may vary for each breast cancer type. For example, hormone therapies are not helpful for Triple-Negative Breast Cancers or using anti-HER2 drugs is not to benefit for Luminal A type breast cancer which tends to be HER2⁻ while this treatment strategy is used for HER2⁺ Luminal B. Also, inappropriate treatment application may cause various side effects or drug resistance development. So, accurate diagnosis and treatment is of vital importance for breast cancer treatment such as other cancer types and development of novel therapeutic approaches or anti-cancer agents is needed and important for fighting against cancer.

Autophagy plays a survival role for normal cells because of being a mechanism which maintains energy homeostasis and is a source of nutrition and an elimination mechanism of damaged organelles and proteins. However, autophagy plays also important roles in carcinogenesis. In a tumor microenvironment, which contains stress conditions such as hypoxia, oxidative stress and nutrient deprivation, autophagy provides survival conditions by maintaining biomolecules and energy for cancer cells and causes adaptation of cancer cell to tumor microenvironment, therefore autophagy plays a role as a tumor promoting oncogenic mechanism [40,41]. But, on the other hand, autophagy has a tumor suppressor role because of maintaining genomic stability, elimination of reactive oxygen species (ROS), degradation of oncogenic proteins and induction of immunoresponse. Bidirectional role of autophagy in tumorigenesis depends on type and stage of cancer [42]. Thus, relationship between autophagy and cancer still has been controversial. Among the programmed cell death mechanisms, especially apoptosis has been widely studied in cancer biology. The first studies suggesting that apoptosis blocks tumor progression by elimination of malignant cells were performed in 1970s [43]. The mechanisms which cause dysregulation of apoptosis and promote tumor progression can be summarized as disruption of the balance between pro-apoptotic and anti-apoptotic proteins, distortion of death receptor signals and reduction of caspase function [43]. Cancer cells generally tend to evade from apoptosis and thus, they may gain capability of escape from immun system surveillance. In short, it is known that programmed cell death mechanisms are the first barriers preventing proliferation and survival of cancer cells. Therefore, various cancer treatment strategies targeting programmed cell death mechanisms have been developed and used until

now. The type of programmed cell death which is induced by anti-cancer agent is depend on multiple factors such as type of cancer cell, dose of drugs, tumor microenvironment and type of cellular damage. So, to understand differences between programmed cell death mechanisms may contribute development of novel anti-cancer agents and treatment approaches.

In our previous study, we investigated cellular effects of the DC on lung cancer cells and revealed that the DC induced autophagic cell death in an apoptosis-independent manner in NSCLC cells [44]. In this study, we aimed to investigate the biological effects of the DC in breast cancer according to metastatic features of cancer cells. We performed our experiments by using two different breast cancer cell lines, MDA-MB-231 and MCF-7 because of being cell culture models of different subtypes of breast cancer. MCF-7 cell line is estrogen receptor, progesterone receptor and HER2 positive, while MDA-MB-231 is negative. So, Luminal A breast cancer subtype MCF-7 is more appropriate cell line model for hormone therapy studies while triple-negative breast cancer subtype MDA-MB-231 cell line is generally used for chemotherapy and radiotherapy studies. MCF-7 cell line is a non-metastatic, but MDA-MB-231 is a highly metastatic cell line because of overexpression of EMT markers (Comşa et al., 2015; Subik et al., 2010).

Firstly, we examined the anti-proliferative effects of the DC on both breast cancer cell lines and determined that the DC suppressed proliferation of both cell lines. Depending on our previous study which showed that the DC induced autophagic cell death in NSCLC cell lines, we thought that the DC may induce programmed cell death mechanisms in breast cancer cell lines and we firstly evaluated protein expression levels of LC3BII for autophagy determination. We observed that autophagy was induced in MDA-MB-231 cell line during 48 hours, but it was only induced for the first hour and then it was reduced gradually during 48 hour-treatment in MCF-7 cell line (Fig. 3). This result showed that the DC induces a non-autophagic cell death mechanism in MCF-7 cell line unlike MDA-MB-231 cells. Depending on this result, we had two question; which cell death mechanism was induced by the DC in MCF-7 cell line and why these two breast cancer cell lines chose different cell death mechanisms? Therefore, we performed TUNEL assay for both the DC-treated cell lines and we obtained more signals in MCF-7 as compared to MDA-MB-231, indicating that MCF-7 underwent apoptosis process (Fig. 4a). To validate these results, we also evaluated protein expression levels of anti-apoptotic BCL-2 and pro-apoptotic BAX. The results which showed that the DC induced apoptosis in MCF-7 but not MDA-MB-231 cell line verified TUNEL assay results (Fig. 4b,c). Also, the DC reduced the invasive capability of metastatic MDA-MB-231 cells (Fig. 5). Upon this results, we wanted to elucidate the mechanisms which cause selection of different cell death pathways of breast cancer cell lines. Therefore, we hypothesized that the different cellular and genetic characteristics between the two cell lines may be responsible for these results. As is known, MCF-7 cell line is p53-mutant while MDA-MB-231 is p53-wild type. Tumor suppressor p53 is a key regulator which play important role in cell cycle arrest, maintaining cellular genomic integrity and controlling cell growth, senescence, differentiation, and apoptosis [47]. p53-mediated growth inhibition depends on induction of an inhibitor of cyclin-dependent kinases p21 and p21 provides a functional link between p53 and cell cycle control [48,49]. Therefore, we evaluated protein expression levels of p53, p21 and another cell cycle and cell death regulator c-Myc. The results showed that p53 expression levels had not any significant change, p21 expression was induced relatively in both the DC-treated cell lines. But interestingly, c-Myc

expression was induced significantly in MDA-MB-231, while it was downregulated in MCF-7 upon the DC treatment (Fig. 6). Next, to determine whether autophagy has an effect on the DC-induced c-Myc inhibition, we treated MCF-7 and MDA-MB-231 with Torin1 and chloroquine, an inducer and a blocker of autophagy process, respectively. Chloroquine is a widely used autophagy inhibitor in the last stage of process by increasing lysosomal pH and blocking lysosome-autophagosome fusion [50]. Torin1 prevents phosphorylation of downstream targets of mTORC1 and so induces autophagy [51]. When we treated MDA-MB-231 cell line with the DC under autophagy-blocked conditions by chloroquine, we observed that c-Myc expression was downregulated significantly whereas c-Myc expression was upregulated upon only the DC treatment under normal conditions (Fig. 7b). Also, we induced autophagy with Torin1 in MCF-7 cell line and under autophagy-induced conditions the DC treatment could not repress c-Myc expression (Fig. 7a). According to these results, the DC downregulates c-Myc following induction of apoptosis in MCF-7 cells while it upregulates c-Myc following induction of autophagy in MDA-MB-231 cells. However, this is a recoverable mechanism via regulation of autophagy in both cell lines and we thought that there is a mutual interaction between autophagy and c-Myc. Because, MCF-7 has low expression level of c-Myc as compared to MDA-MB-231 [52,53]. In many studies, it was reported the interaction between autophagy and c-Myc (Z. Chen et al., 2019; Toh et al., 2013). Also, it was showed that Apigenin combined with Gefitinib blocks autophagy by inducing apoptosis through inhibition of c-Myc [56]. Recent studies showed that c-Myc overexpression promotes EMT by downregulating E-cadherin expression in breast cancer (Cho et al., 2010; Gao et al., 2019). EMT is a process that cells lose their epithelial phenotype and acquire mesenchymal features and it was reported that there is a crosstalk between autophagy and EMT in various cancer types in recent studies (H. T. Chen et al., 2019; Gugnoni et al., 2016). As previously mentioned, MCF-7 is a non-metastatic cell line and has an epithelial character while MDA-MB-231 has mesenchymal phenotype and highly metastatic capacity. Depending on this difference, we thought that metastatic features and expression levels of EMT markers of cell lines may determine the response to the DC, hence the cell death mechanism type. Therefore, we performed plasmid transfection for overexpression of Twist in MCF-7 cell line. Because low endogenous Twist expression is observed in MCF-7 as compared to MDA-MB-231 cell line and Twist upregulation may cause induction of EMT, invasion and migration of breast cancer cells [65]. As a result of our analysis, we observed overexpression of c-Myc in exogenous Twist expressing MCF-7 cells treated the DC similar to MDA-MB-231 cell line (Fig. 8).

In recent studies, it was reported that cancer cells may induce autophagic cell death mechanism as a response to anti-cancer agents [66–68]. But, it is still unclear that clinic effects of autophagy on cancer. Therefore, as target mechanisms, selection of the distinctive mechanisms between normal cells and cancer cells may be a useful approach for development of anti-cancer drugs. In this study, we determined that the DC induces autophagic cell death in metastatic breast cancer but it induces apoptotic cell death non-metastatic breast cancer. This different death pathway selection of different cancer cell lines may occur Twist/c-Myc axis, depending on metastatic features of breast cancer cells.

In conclusion, taken together with our previous study, the DC has anti-proliferative effects on lung and breast cancer and causes cell death. But determination of cell death mechanism type is depend on

cancer cell type, even sub-type. Therefore, further studies is needed to illumination of detailed mechanisms of cellular effects of the DC and distinctive mechanisms between several cancer types. We hope that our findings may provide a novel perspective for interactions of autophagy, apoptosis and EMT pathways and may contribute to further analysis for development of novel anti-cancer therapies.

Abbreviations

DC: the triazole linked galactose substituted dicyano compound

LC3B: Light chain 3 B

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

MTT: Methylthiazolyldiphenyl-tetrazolium bromide

BAX: Bcl-2 Associated X Protein

BCL-2: B-cell lymphoma 2

DC: The dicyano compound (the triazole linked galactose substituted dicyano compound)

Declarations

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Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Consent for publication: The authors hereby consent to publication of this study.

Compliance with Ethical Standards

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Figures

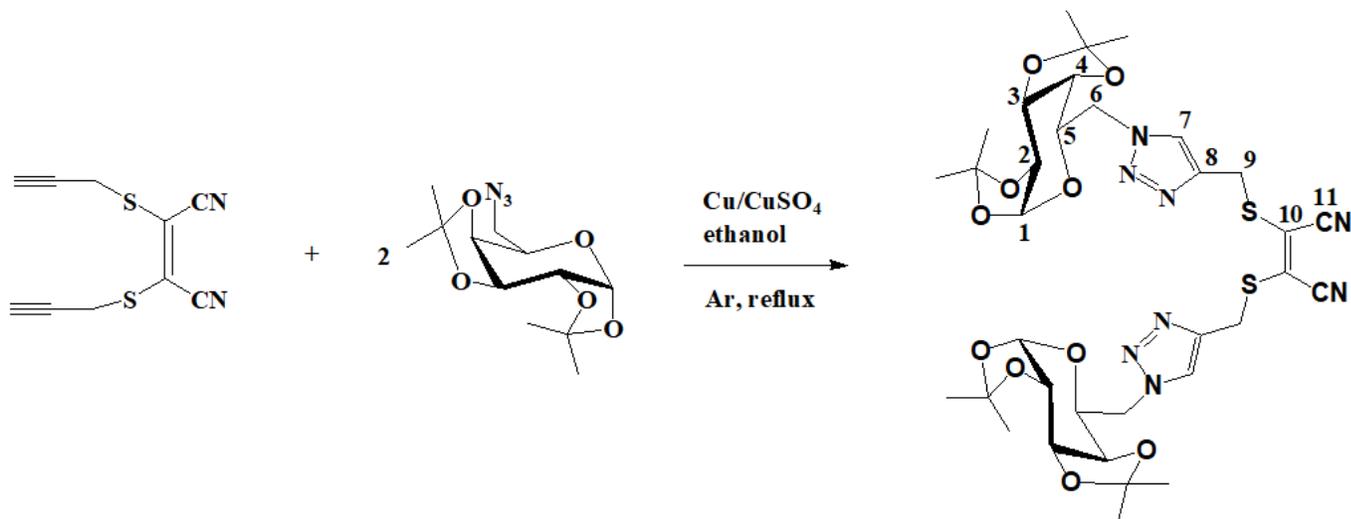


Figure 1

The synthesis stages of the DC and its chemical structure

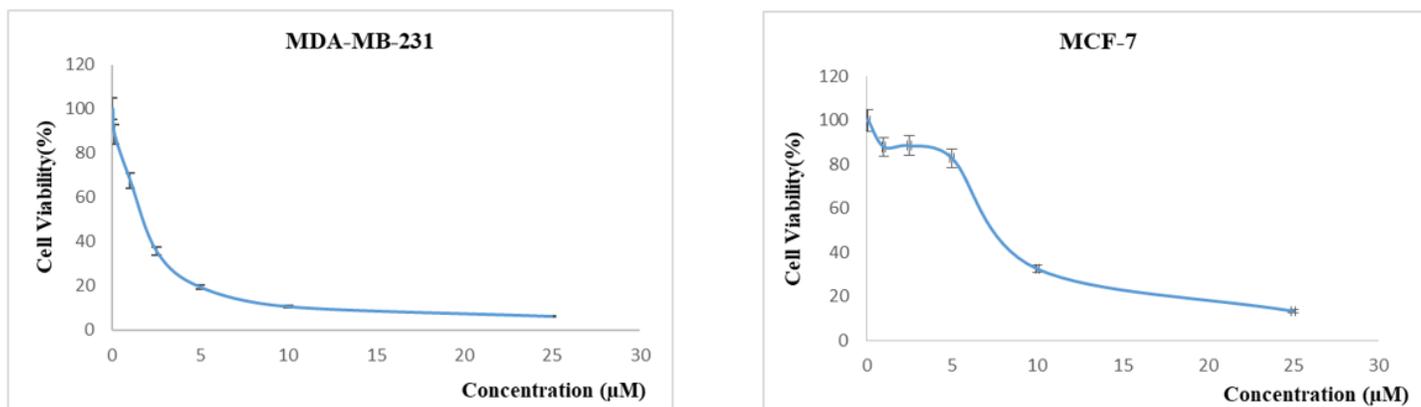


Figure 2

The DC has an anti-proliferative effect on breast cancer cell lines. The DC caused a significant decrease in cell viability of both cell lines as compared to untreated control samples as a dose-dependent manner. a: Proliferation graph of treated MDA-MB-231 cell line. b: Proliferation graph of treated MCF-7 cell line. (The graphs show the means±S.D. of a representative experiment performed in independent triplicate.)

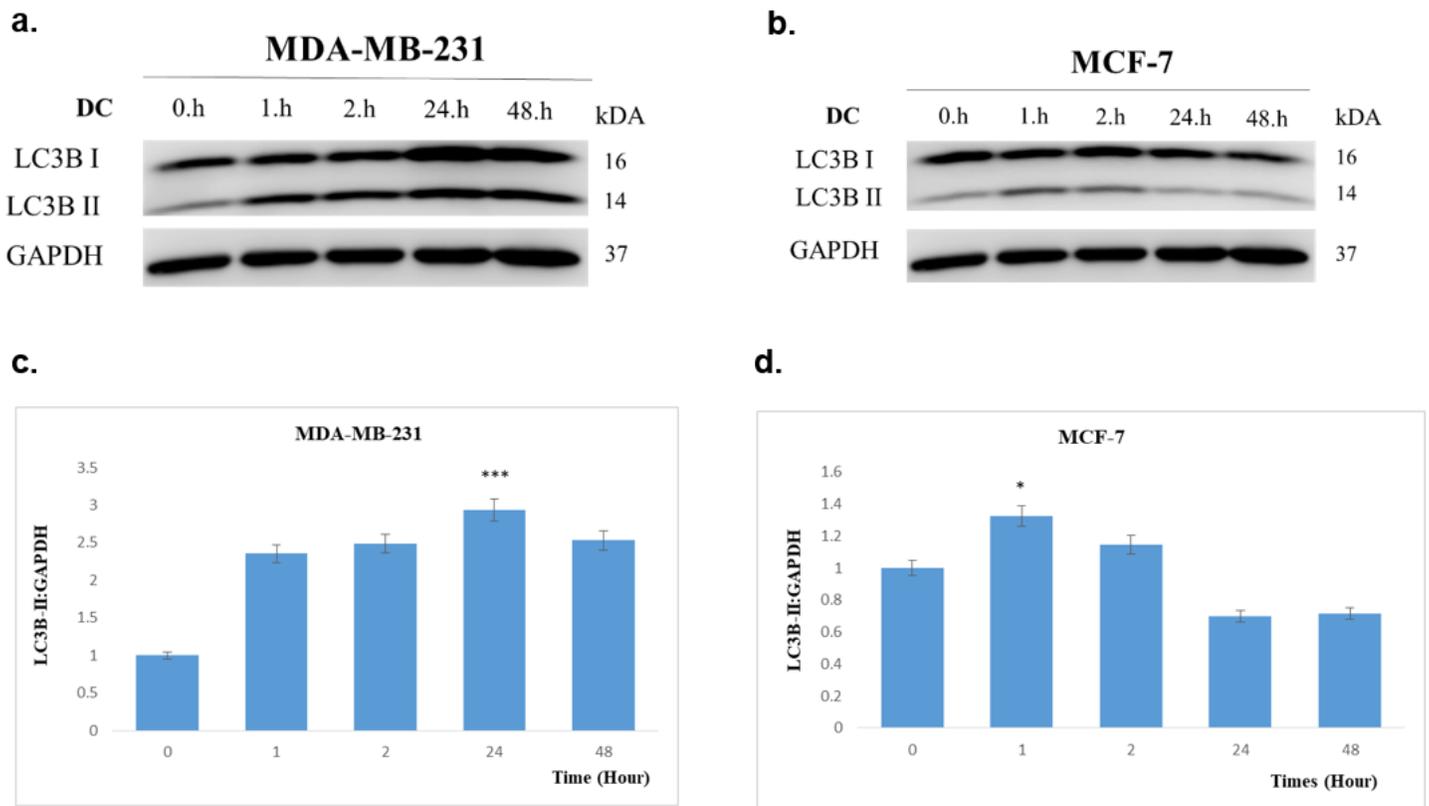


Figure 3

The DC induced autophagy in metastatic MDA-MB-231, but not in non-metastatic MCF-7 as long-term manner. a, b) Protein expression of LC3B of 48-hour the DC-treated MDA-MB-231 and MCF-7 cell lines, respectively. (DC: the dicyano compound) c, d) The graphs depicting quantification of LC3BII:GAPDH ratios in the experimental set-up shown in a and b, respectively. (* $p < 0.05$, ** $p < 0.03$, *** $p < 0.01$ in comparison with the untreated group. The graphs show the means \pm S.D. of a representative experiment performed in independent triplicate.)

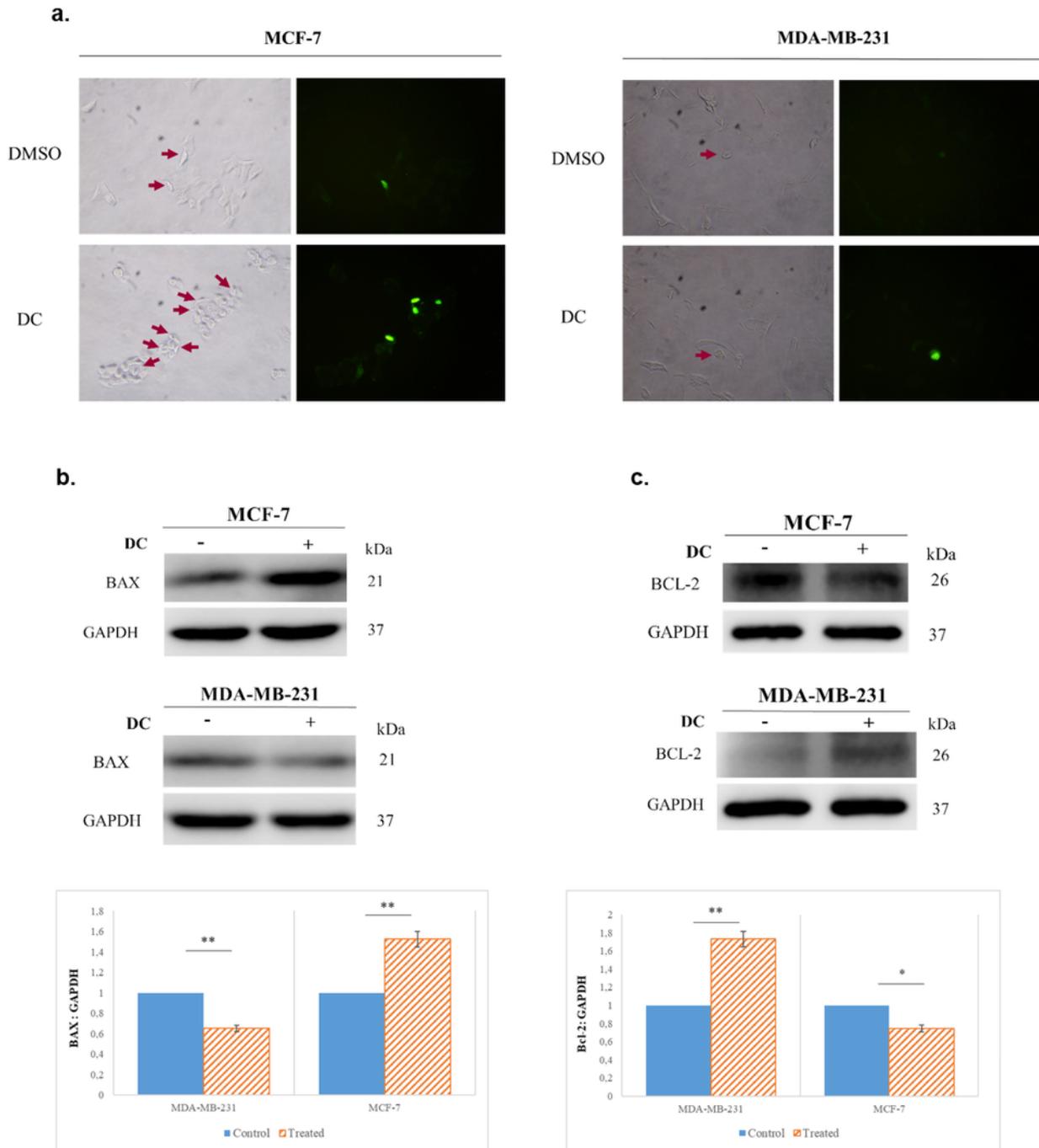


Figure 4

The DC induced apoptosis in non-metastatic MCF-7, not in metastatic MDA-MB-231 cell lines. Determination of apoptosis in 48-hour the DC-treated MCF-7 and MDA-MB-231 cell lines by TUNEL a) Brightness and 480 nm wavelength phase image of control group and the DC-treated group of MCF-7 and MDA-MB-231 cell lines, respectively. b, c) Immunoblots of pro-apoptotic BAX and anti-apoptotic Bcl-2 in 48-hour DC-treated MCF-7 and MDA-MB-231 cell line and the graphs depicting quantification of the

protein levels. (DC: the dicyano compound) (* $p < 0.05$, ** $p < 0.03$, *** $p < 0.01$ in comparison with the untreated group. The graphs show the means \pm S.D. of a representative experiment performed in independent triplicate.)

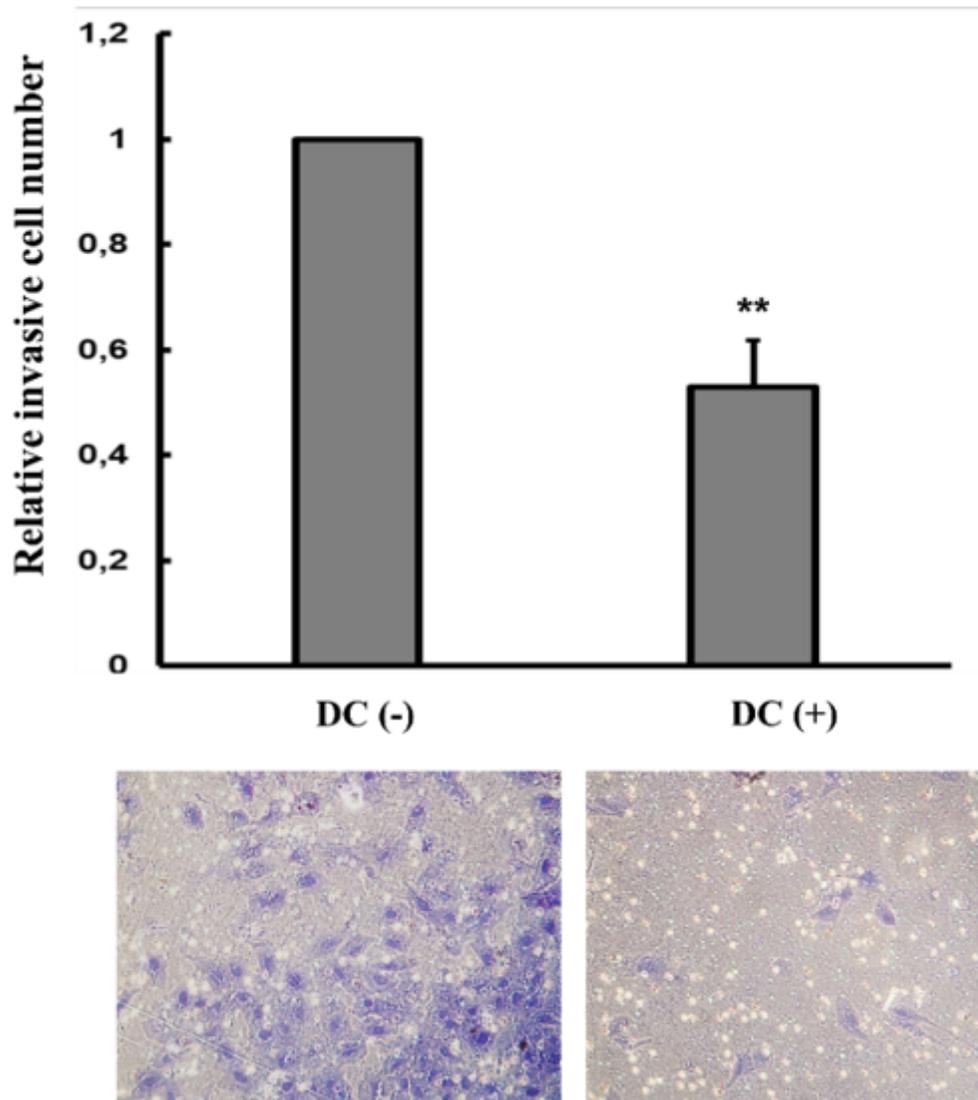


Figure 5

The DC reduces invasive capability of metastatic MDA-MB-231 cell line. Invasion capability of MDA-MB-231 cells untreated and treated with the DC during 48 hours, respectively (* $p < 0.05$, ** $p < 0.03$, *** $p < 0.01$ in comparison with the untreated group. The graphs show the means \pm S.D. of a representative experiment performed in independent triplicate.)

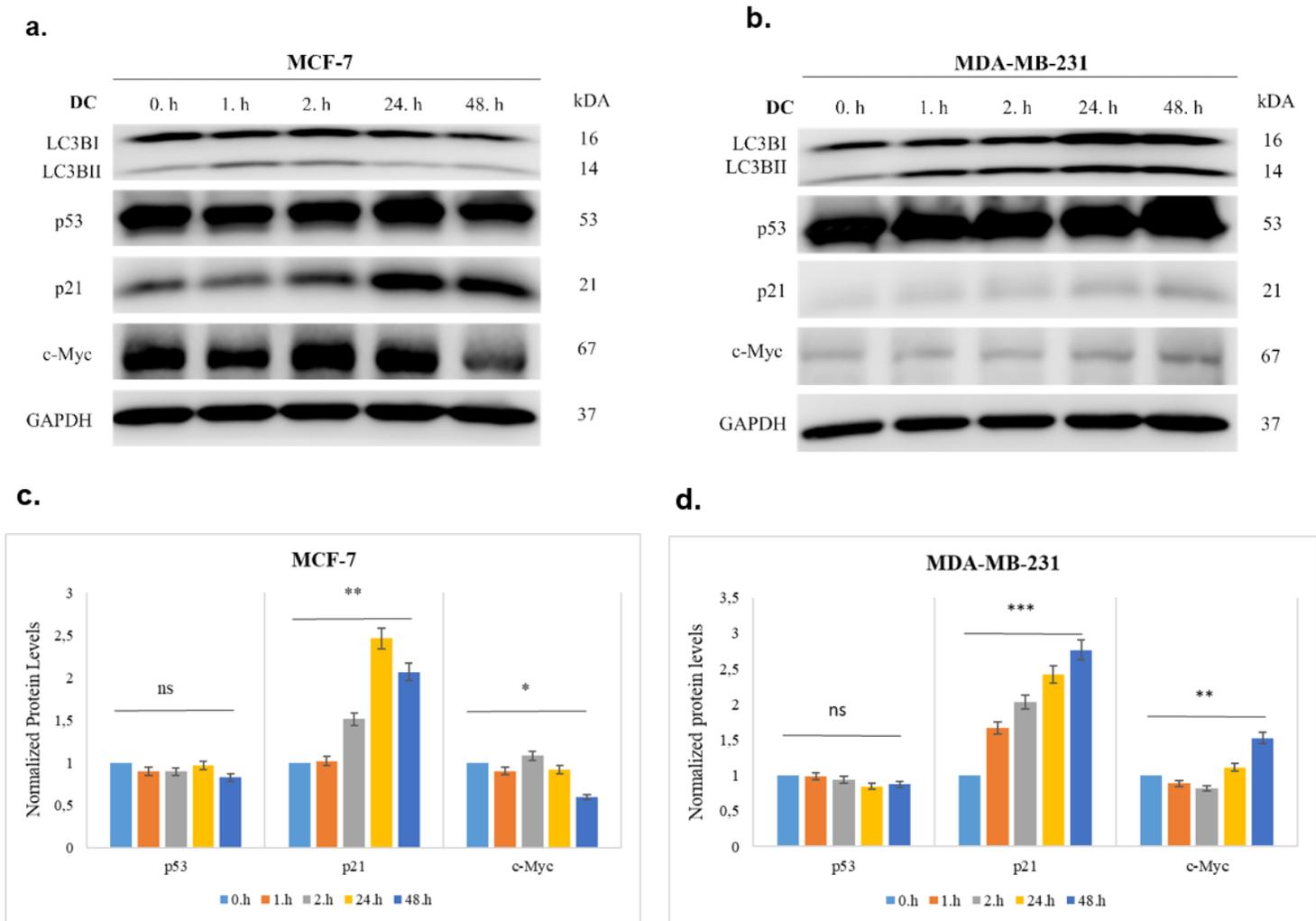


Figure 6

The DC regulated c-Myc and p21 expression levels as a p53-independent manner. a, b) Immunblots of p53, p21 and c-Myc protein in MCF-7 and MDA-MB-231 cell lines, respectively. c, d) The bar graphs represent normalized expression levels of respective proteins in the experimental set-up shown in a and b, respectively. (* $p < 0.05$, ** $p < 0.03$, *** $p < 0.01$ in comparison with the untreated group. The graphs show the means \pm S.D. of a representative experiment performed in independent triplicate.)

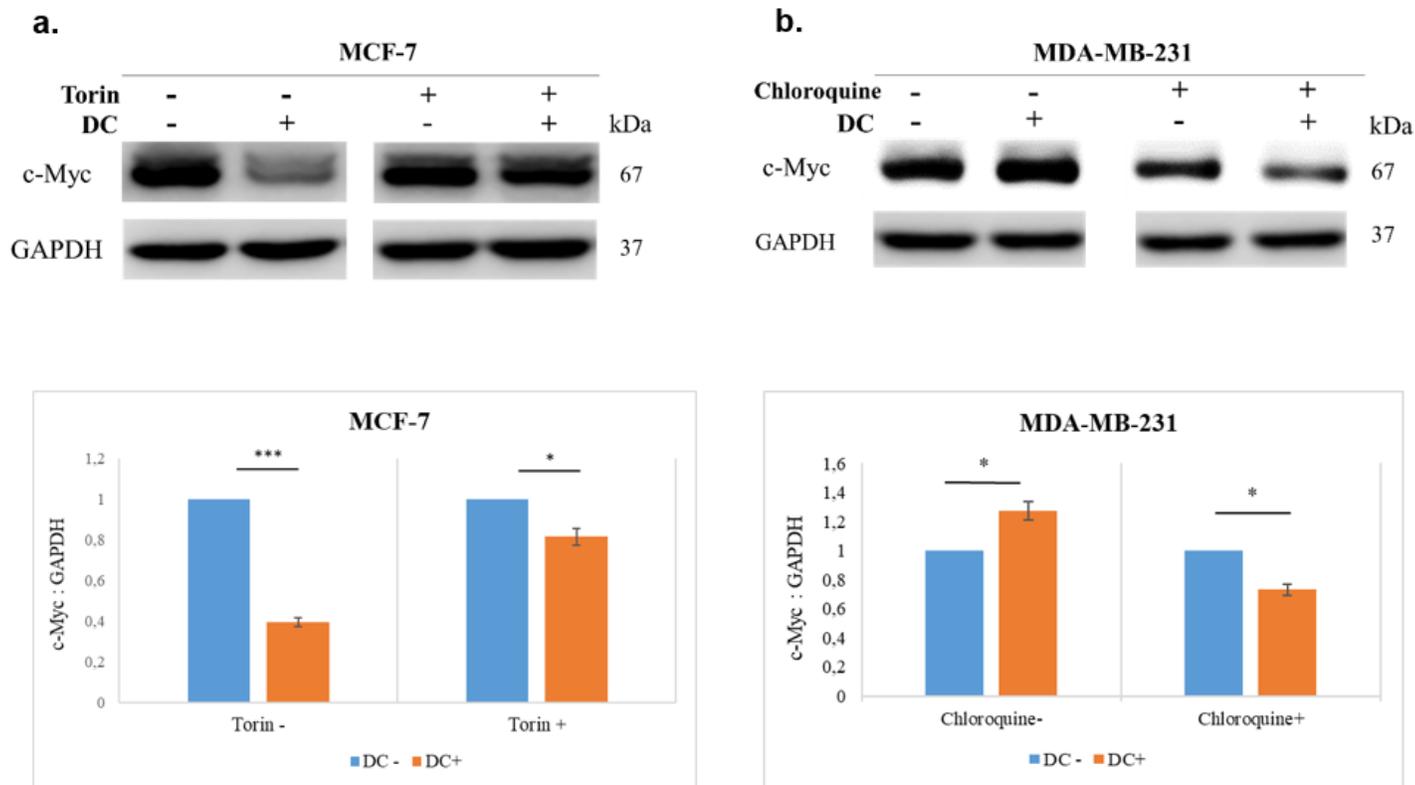


Figure 7

c-Myc may play role in selection of cell death pathway following the DC. Immunblots of c-Myc protein in MCF-7 cell line in which is autophagy was induced by Torin (a) and in MDA-MB-231 cell line in which is autophagy was blocked by chloroquine (b) The bar graphs represent quantification of c-Myc:GAPDH ratios in the experimental set-up shown in a and b, respectively (* $p < 0.05$, ** $p < 0.03$, *** $p < 0.01$ in comparison with the untreated group. The graphs show the means \pm S.D. of a representative experiment performed in independent triplicate.)

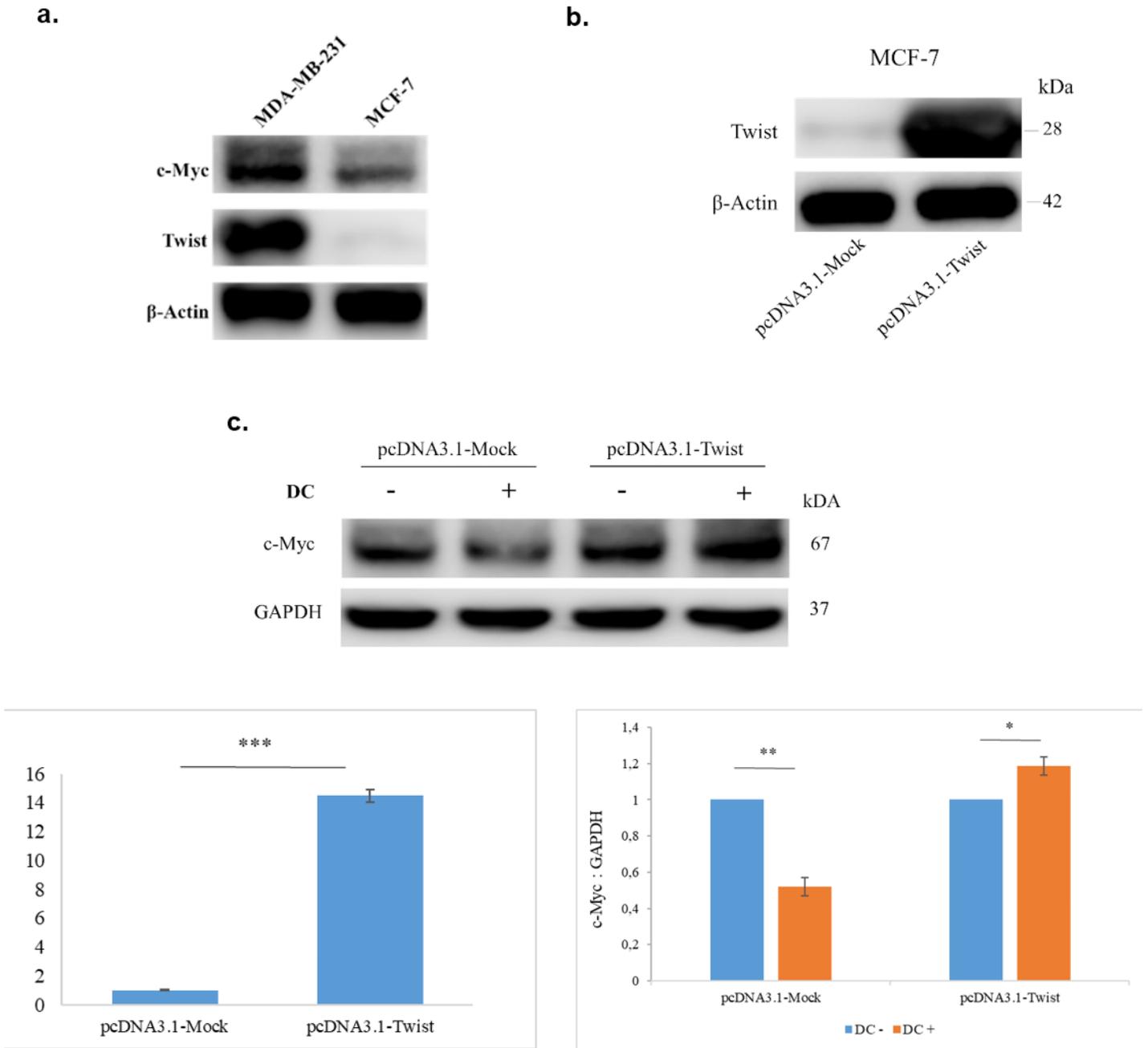


Figure 8

Twist abolished the DC-induced c-Myc downregulation in MCF-7 cell line. (a) Immunblots of c-Myc and Twist proteins in MDA-MB-231 and MCF-7 untreated cell lines (b) Immunblots of c-Myc protein in MCF-7 cell line which was transfected by pcDNA3.1-Mock and pcDNA3.1-Twist plasmids (c) The bar graphs represent normalized expression levels of Twist and c-Myc in the experimental set-up shown in b and c, respectively. (The graphs show the means \pm S.D. of a representative experiment performed in independent triplicates.)

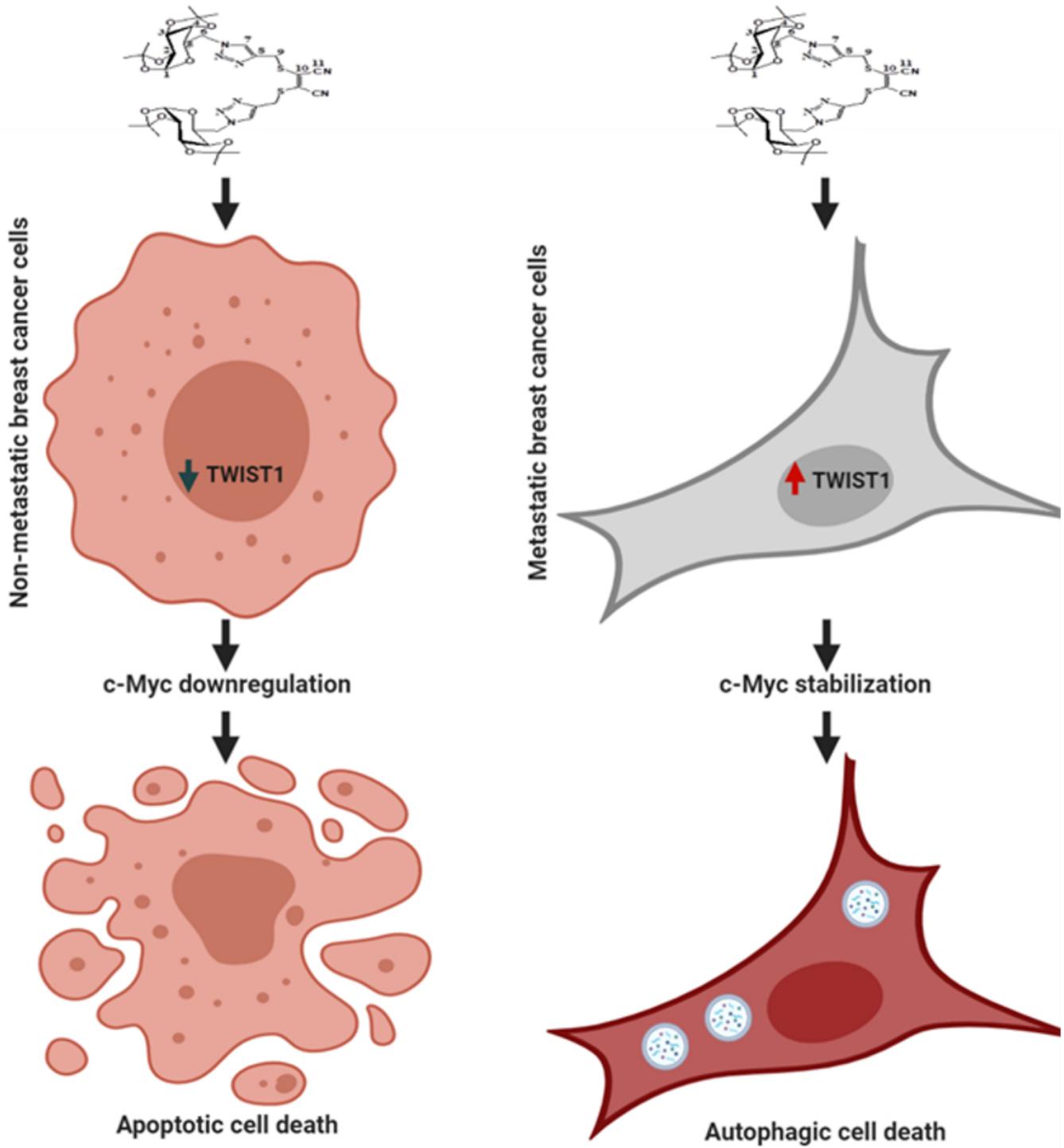


Figure 9

The schematic representation of the results.