Inflated endoplasmic reticulum and autophagy induction promoted death in aggressive cancer cells grown in adherent and in-vitro CTC conditions.

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Abstract

Cancer metastasis is the leading cause of death in cancer patients and is a major challenging aspect of cancer biology. Various adaptive molecular signaling pathways play a crucial role in cancer metastasis and later in the formation of secondary tumors. Aggressive cancer cells like Triple Negative breast cancer (TNBCs) are more inclined to undergo metastasis hence having a high recurrence rate and potential for secondary tumor formation. Such aggressive cancer cells have fewer treatment options available with poor prognoses. Metastatic cells in circulation known as circulating tumor cells (CTCs) could be an appealing alternative target for cancer therapy with potentially better outcomes. Cell cycle regulation and stress response of CTCs in blood has a crucial role in their survival and progression and thus may be considered therapeutically active hotspots. The cyclin D/cyclin-dependent kinase (CDK) pathway regulates cell cycle checkpoints, a process that is frequently dysregulated in cancer cells. Selective CDK inhibitors can limit the phosphorylation of cell cycle regulatory proteins by inducing cell cycle phase arrest, and thus may be an effective therapeutic strategy for aggressive cancer cells in their dividing phase at the primary or secondary site. However, during the floating condition, cancer cells halt their division process and proceed through the various steps of metastasis. Our study showed that a novel CDK inhibitor 4ab besides showing anti-proliferative activity in aggressive cancer cells also induces cell death by activating endoplasmic reticulum stress and strong autophagy induction in both adherent and floating conditions which resulted in cytoplasmic vacuolation (paraptosis) and ultimately resulted in cell death. Further our results showed that 4ab efficiently induced apoptosis in aggressive cancer cells by initiating the ER-mitochondrial membrane potential loss-JNK pathway. Further, our in vivo data showed a promising inhibitory effect of 4ab on aggressive 4T1 cells in tumor formation and metastasis. The above data gave us encouraging results to consider 4ab as a potential inhibitor for anti-metastatic therapeutic intervention.

Introduction

Cancer is the unchecked proliferation of cells and occurs mostly by dysregulation of proteins governing the cell division which mainly include cyclin-dependent kinases (CDKs) [1–3]. CDKs are multifunctional kinases having a role in regulating various indispensable pathways of cell-like cell division, transcription and differentiation [4, 5]. CDKs/cyclins act in concert and their function is dysregulated in various pathological conditions for example in the range of human cancers, neurodegenerative disorders, renal diseases, and inflammation [6–15]. The cell cycle is mainly regulated by CDK 1, 2, 3, 4 and 6 and their respective cyclins A, B, C, D, and E, while transcription is regulated by 7, 8, 9 and 11 CDKs and their corresponding cyclins are C, H, L, and T [16]. In most malignancies, CDK dysregulation is often the reason for their aggressiveness and thus targeting CDKs in tumor cells has become a promising therapeutic strategy [17]. CDK/Cyclin regulation was shown to play a crucial role in supporting anoikis (detachment-induced cell death) resistance and metastatic progression [18]. More recently, CDK4/6 inhibitor palbociclib was licensed by the Food and Drug Administration (FDA) for the treatment of metastatic breast cancer [19]. Most human cancer cells and patient tissue have high levels of CDK mRNA and
protein. Many studies have established that inhibition of different CDKs decreases cancer cell growth, modulates autophagy and induces apoptosis [20–23]. Autophagy is an intracellular stress-activated catabolic process in which two components- autophagosome and lysosomes play an important role in it [24, 25]. Autophagy can act as pro or anti to cancer progression and is context-dependent, however during metastasis autophagy supports survival and the dissemination of aggressive cancer cells like TNBCs [18]. Aggressive cancer types like pancreatic ductal adenocarcinoma (PDAC) and TNBCs, impose a grand challenge in oncology due to late-stage diagnosis and low survival rate [26, 27]. The only potential cure for PDAC and TNBCs is the surgical removal of cancer followed by random chemotherapy, though early cancer cell dissemination remains a major obstacle clinically [28–33]. Anticancer drugs e.g gemcitabine, and nab-paclitaxel, etc are usually toxic with serval off-targets having a spectrum of side effects [34–36]. Hence, advanced therapeutic schemes with minimum toxicity, and better efficacy in inhibiting cancer progression and death induction are needed. CDKs have been projected as pharmacologically efficient targets in several malignancies. But resistance shown by cancer cells is the cause of failure of these drugs in the long run treatment. Our previously reported inhibitor 4ab was effective to activate the death pathway specifically in aggressive cancer cells. 4ab induced endoplasmic reticulum (ER) stress mediated autophagy and apoptosis activation in highly invasive cancer cells in both adherent and floating conditions. Moreover, our in vivo data demonstrated that 4ab was effective in decreasing lung metastatic burden in the syngenic mouse model. Together above findings uncovered that CDK inhibitor 4ab induce death in aggressive cancer cells by ER stress-activated death pathways and thus can be effective to withstand the cancer adaptive development of drug resistance and consequently, is a promising small molecule for the development of a new anti-cancer drug.

Results

4ab induces death in triple-negative breast cancer by cytosolic vacuolation and induction of autophagy.

Our earlier report demonstrated that 4ab showed cytostatic potential on various cancer cells by inhibiting CDK9 and CDK2 [37]. In the present study, we tried to check the potency of 4ab on aggressive triple-negative breast cancer cells in attached and detached conditions. Our results demonstrated that 4ab was effective in inducing cell death in TNBCs in both conditions as shown in Figure 1 A. Later 4ab treated TNBCs (MDA-MB-231 and SUM-159) cells were processed for morphological assay which demonstrates the vacuole-like structure formation at concentrations of 0, 2, 4, 8, and 16 µM indicating the paraptotic death induction (Fig. 1B). Lysates from attached and detached TNBCs treated with 4ab at concentrations 0, 1, 2, 4, 8 and 16 µM for 24 h were processed for western analysis. Our western results showed that the expression of LC3-II was upregulated and p62 was downregulated in a concentration-dependent manner indicating the strong autophagy activation in these cancer cells (Fig. 1C, D).

4ab strongly inhibits the PI3K/AKT/mTORC1 pathway in both attached and detached conditions of TNBCs.
MDA-MB-231 cells were treated with 4ab in a concentration-dependent manner (0, 1, 2, 4, 8, 16 µM) in both attached (Att.) and floating (Dett.) conditions and processed for protein expression by western blotting. Our results showed that 4ab inhibits the expression of PI3K, p-AKT, and p-mTORC1 significantly which resulted in the overactivation of the autophagy (type-II programmed cell death) process in TNBCs in both conditions (Fig. 2A-D).

4ab induces ER stress in highly aggressive TNBCs in adherent and floating conditions.

In view of the induction of massive vacuole formation (~ER dilation) upon 4ab treatment in MDA-MB-231 cells immediately (3 h) (Fig. 3A-C). We performed time-dependent (0, 3, 12, 24 h; 16 µM) and concentration-dependent (0, 1, 2, 4, 8, 16 µM; 24 h) treatment of 4ab to MDA-MB-231 cells in attached condition and later were analysed for western blotting. Our results showed that 4ab treatment resulted in the formation of cytosolic vacuolization and overexpression of the ER stress response proteins or (UPR), ATF6, IRE1-alpha and PERK. Further 4ab treatment to MDA-MB-231 in a detached condition also induced ER stress in a time-dependent manner. Expression of BiP was downregulated by 4ab indicating sustained induction of UPR, which subsequently leads to cell death of aggressive cancer cells both in attached and detached conditions.

4ab activated the intrinsic and JNK pathway-mediated apoptosis in MDA-MB-231 cells.

We next tried to find the effect of 4ab on Mitochondrial membrane potential (MMP) and the expression of proteins associated with it. By using fluorescence microscopy, we observed that 4ab induced MMP loss in a concentration-dependent manner which later activates ROS levels in aggressive cancers in both adherent and floating conditions (Fig. 4A, B; Supplementary Figure 1A). The later effect of MMP loss on the expression of downstream stress sensor (JNK), proapoptotic (Bax) and death proteins (Caspase-3, PARP-1) was analyzed using western blotting. Our results demonstrated that expression of p-JNK, Bax, apoptotic initiation factor Cytochrome c along with cleavage of Caspase-3 and PARP-1 was enriched in the whole-cell lysate (Fig. 3C, D). Collectively, the above data suggested that 4ab is reducing MMP by activating intrinsic pathways and upregulating p-JNK expression.

4ab induced cell death in aggressive pancreatic adenocarcinoma (MIA PaCa-2) via JNK-mediated autophagy and apoptosis activation.

After confirming that 4ab effectively induced death in TNBCs. We tried to find its effect on pancreatic adenocarcinoma cells. MIA PaCa-2 cells were treated with 4ab with concentrations (0, 0.125, 0.25, 0.5, 1 and 2 µM) and were processed for microscopy and western blot analysis. Our microscopy results showed that 4ab was also responsible for the vacuolation which increased at higher treatment conditions (Fig. 5A). Moreover, the expression of stress to death sensor (JNK), proapoptotic protein (Bax) and cleavage caspase-3 and PARP-1 was upregulated by 4ab in concentration-dependent manner. Our western results also revealed that 4ab strongly activated complete autophagy by increasing LC3-II and decreasing P62 expression (Fig. 5B, C).
**4ab leads to the loss of invasion, migration and colony formation potential in TNBCs**

We later tried to identify the effect of 4ab on the metastatic potential of the aggressive cancer cell (TNBC). TNBCs were incubated with 4ab (0, 1, 2, 4, 8 and 16 µM) and analyzed microscopically for their effect on invasion, colony formation and migration potential. Our microscopic results confirmed that 4ab significantly reduced the invasive and migratory potential, besides, colony formation of TNBC was drastically reduced in presence of 4ab even at lower concentrations (Fig. 6A-F). Moreover, 4ab was effective in reducing the promoters of epithelial-mesenchymal transition by downregulating TWIST-1 expression and subdued stemness by decreasing the expression of SOX-2 and mammosphere formation in TNBCs **Supplementary Figure 2**.

**4ab failed to induce autophagy and apoptosis in non-aggressive cancer cells (MCF-7)**

We tried to find the effect of 4ab on non-aggressive cancer cells like MCF-7 (hormone positive). Cells after treatment with 4ab (0, 2, 4, 8, 16 and 32 µM) were processed for microscopy and western analysis to check morphological and protein expression changes. Our results validated that 4ab was not inducing vacuole formation (Fig. 7A). Moreover, 4ab was not inducing autophagy and apoptosis in non-aggressive cancer cells (Fig. 7B). This depicts that 4ab is specifically targeting the aggressive cancer cells and decreasing their proliferative and invasive potential.

**4ab decreases the tumor expansion in the syngenic tumor model**

Since our in vitro data of 4ab showed promising results in decreasing the invasiveness of highly aggressive cancer cells, so we tried to validate the results in the syngenic BALB/C model. Our results demonstrated that 4ab at 5 and 10 mg/kg, decreased the tumor formation potential and dissemination of 4T1 cells and reduced the colonization in the lungs compared to the control group (Fig. 8A). Western blotting results also demonstrated that the ER stress proteins (PERK), death initiating stress sensor (p-JNK) and autophagy marker protein (LC3-II) were upregulated in 4ab treated mice compared to vehicle group as shown **Figure 8B, C**. Above data supported that 4ab was efficiently reducing the tumor burden and metastatic potential of aggressive 4T1 cells.

**Graphical representation of 4ab: image representing the effect of 4ab on death-inducing pathways in aggressive cancer cells. 4ab induces ER stress and activates autophagy which induces vacuolation in the cytosol of aggressive cancer cells causing death by activating apoptosis.**

**Discussion**

Triple-negative breast cancer and pancreatic adenocarcinoma are highly invasive cancer and have poor therapeutic responses [38–41]. These cancer types spread faster and have limited therapeutic options available. As these cancer cells are clinically aggressive and require adjuvant chemotherapy to improve survival. Patients with TNBC or pancreatic adenocarcinoma malignancy have a high risk of earlier metastasis and the chance of survival is comparatively less within 5 years of diagnosis. Neoadjuvant
chemotherapy is highly recommended for these patients, but due to its toxic nature for normal cells and less significance to patients with residual disease. These patients have a higher chance to undergo cancer metastasis and an overall shorter life span compared to patients with less aggressive tumors. Since metastasis is the cause of 90% of cancer-related death [18]. The complexity of metastasis is causing the paucity of therapeutic options since metastasis is a multistep process and each step is controlled by unexplored multi factors resulting in the potential molecular targets remaining to be defined [18]. In this study, we have shown that 4ab besides its CDK inhibitory activity, was also responsible for the activation of alternative death pathways in highly aggressive cancer cells. 4ab treatment immediately leads to cytosolic vacuolization of aggressive cancer cells. A spectrum of studies has shown that various stress insults resulted in endoplasmic reticulum swelling and overactivation of autophagy that collectively results in cytosolic vacuole formation mediated cell death called paraptosis [42–48]. The present study has demonstrated that 4ab at higher concentrations strongly activates autophagy and ER stress or unfolded protein response (UPR) in both aggressive cancer cells grown in attached and floating conditions by inhibiting PI3K/AKT pathway. Our results further revealed that 4ab activated the stress (ROS) mediated JNK pathway which ultimately resulted in apoptosis in TNBC and PDAC by mediating Caspase3 and PARP-1 cleavage. Since aggressive cancer cells are highly metastatic and show higher invasiveness, migratory potential and higher frequency for colony formation [49, 50]. Our study demarcated that 4ab was very efficient in reducing the metastatic and colony formation potential in aggressive cancer cells. Various reports suggest that cancer stem cells (CSCs) are responsible for cancer metastasis and progression [51–54]. Related to that, our findings showed that 4ab effectively reduced the mammosphere formation and decreased CSC maker; in addition to that 4ab reduced the epithelial-mesenchymal marker thus acting as an effective metastatic inhibitor. Meanwhile, our study further revealed that 4ab was less effective in inducing the death of non-aggressive cancer cells (MCF-7). Additionally, our data showed that 4ab failed to induce UPR and autophagy-mediated cell death in MCF-7 cells which indicated the specificity of this small molecule towards aggressive cancer cells. Our in vivo results also demonstrated that 4ab was active in reducing the metastatic potential of 4T1 cells by decreasing the lung tumor nodule formation by increasing the expression of ER stress and autophagy marker proteins.

In conclusion, 4ab acts as a potent analog against multiple targets like cyclin-dependent kinase, and pathways like autophagy and apoptosis. Besides, 4ab promoted death in anoikis-resistant cancer cells (TNBC and PDAC) in both adherent and floating conditions significantly thus acting as an anti-metastatic molecule. 4ab also showed a versatile pharmacokinetic profile and antitumor activity in in-vivo (syngenic mice model). With this supporting data, 4ab can be considered having therapeutic active molecule, specifically for highly aggressive cancer cells.

**Materials And Methods**

**Chemicals, reagents and antibodies:**

Chemicals, reagents and antibodies
The chemicals and reagents used in our experiments were: bafilomycin A1 (Baf, Sigma, #19-148) DMSO (#D2650), 2′,7′-dichlorofluorescin diacetate (H2DCFDA) (Sigma, #35845), EDTA (Sigma, #E9884), Phosphate buffer saline (Sigma, PBS, #P5493), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, #M2128), phenylmethylsulfonyl fluoride (PMSF, Sigma, #P7626), Sodium orthovanadate (Sigma, #S6508), developer (Kodak, Sigma, #DA163, #P6557), protease inhibitors cocktail (Sigma, #P8340), PVDF membrane (0.2 µm, BIO-RAD#162-0177), protein assay kit (BIO-RAD, #500-0002), chemiluminescences film (Bioscience, #28906035), protein ladder (BIO-RAD, #161-0374), Amersham™ Hypercassette™ (#RPN11644).

The antibodies used in our experiments were: Cell Signaling Technology (CST): p-mTOR (#2971P, #2974S), m-TOR(#2983S) PARP-1 ( #9542SS), anti-p62 antibody (#5114, #8025S), Caspase-3 (#9662S) Sigma-Aldrich: Anti-LC3B antibody (#L7543), p62 (#P0067), Monoclonal anti-β-Actin antibody (#A3854), anti Rabbit IgG-peroxidase antibody (A9169),

**Cell culture**

**Cell lines, culture medium and growth conditions:**

MDA-MB-231, MCF-7, 4T1, MIA PaCa-2, were acquired from American Tissue Cell Culture (ATCC). SUM-159 was procured from a European collection of cell cultures (ECACC). MIA PaCa-2 was grown in DMEM with 10% FBS, MCF10A was grown in DMEM/F12+other supplements with 5% “Horse Serum” whereas MCF-7, 4T1, were cultured in “RPMI-1640” with 10% Fetal Bovin Serum, HEPES (2.38 g/L), sodium bicarbonate (2 g/L), sodium pyruvate (550 mg/L), β-mercaptoethanol (3.5 μl/L), penicillin (100 units/L) and streptomycin (70 units/L). The cells were kept in a humid incubator at 37°C with 5% CO₂ and 98% humidity. The cells were generally grown in Ultra-low attachment or coated flasks (detached or attached conditions) (Nunc or Corning). Cell lines used in this study are adherent cells. Adherent cells get attached to the base of the dish and they grow in a monolayer culture.

**MTT assay**

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to check cell viability. (MTT) the solution prepared in phosphate buffer saline (PBS; 5 mg/ml) was incubated at 37°C and 5% CO₂ with aggressive cancer cells treated with or without 4ab for 2 hours before termination. Formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The intensity of the color was measured by using a microplate reader (TECAN) to take an absorbance reading at 570 nm.

**Protein isolation, quantification, western blotting and immunoprecipitation**

Various cancer cells treated with 4ab in either attached or detached condition were lysed by RIPA lysis buffer and collected for protein estimation, each step was done at 4°C. A later equal amount of protein from each sample was resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, 162-0177). The membrane was later blocked with 5% non-fat milk and then probed
with target primary antibodies overnight at 4\textdegree{}C and HRP\textsuperscript{+}-tagged secondary antibodies (2h, RT). Western signaling was detected by using chemiluminescence horse reddish peroxidase (HRP) substrates and the signals were captured on X-ray film. The density of the various bands in the western blot was quantified using ImageJ.

**Immunofluorescence microscopy**

Cells were treated with 4ab and later stained with mitochondrial membrane potential detecting dye (TMRE) for 15 minutes before termination. Later cells were washed with PBS thrice and taken for imaging. Ten different fields of each sample were taken and the final results were taken as an average mean of each sample.

**In vivo tumor mice model**

The animals were housed under standard husbandry conditions: 24 ± 2\textdegree{}C temperature, 15-20 complete fresh air changes per hour and 50\%-60\% relative humidity as per the guidelines for the care and use of laboratory animals. 1×10\textsuperscript{6} 4T1 cells were injected in BALB/C mice (syngeneic) and tumor assessment was done after 6 days p.o. On day 32 mice were sacrificed and the tumor and lungs were extracted for further experimentation. Tumor measurements included size, weight and volume, and were taken from different groups. Some tumor samples from each group were frozen for protein extraction, which was later done by using tissue lysis buffer and homogenizer. All animal experimental procedures were carried out following the ethical guidelines for the use of animals in experiments and were conducted in compliance with the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA) and the ARRIVE guidelines. All experiments were approved by the animal house CSIR IIIM. The use of experimental animals in this study was approved by the Ethics and Institutional Animal Care and Use, Committees of the Council of Scientific and Industrial Research-Indian Institute of Integrative Medicine (CSIR-IIIM).

**Declarations**

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**Declaration**

**Ethical approval and consent to participate—**

1. The use of experimental animals in this study was approved by the Ethics and Institutional Animal Care and Use, Committees of the Council of Scientific and Industrial Research-Indian Institute of
Integrative Medicine (CSIR-IIIM) following guidelines of the Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA).

2. I confirm that all methods were carried out "in accordance with relevant guidelines and regulations.

3. I confirm that study was carried out in compliance with the ARRIVE guidelines.

Consent for publication

The content of this manuscript has not been previously published anywhere and is not under consideration for publication elsewhere.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that they have no competing interests.

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Author information

Contribution

F.M. supervised the study; F.M. and S.K designed the experiments; S.K. performed most of the experiments and wrote the paper; S.K and K.F helped in the interpretation of the results; S.K and K.F repeated most of the experiments; S.K draws the graphical abstract; S.K and K.F performed animal experimentation; U.S and P.P synthesized the molecule.

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References


Figures

**Figure 1**

4ab induces vacuolation and lethal autophagy in the attached and detached condition of MDA-MB-231. (A) represents the growth inhibitory effect of 4ab on MDA-MB-231 in attached and detached conditions measured by MTT assay after 24 h. (B) shows the morphological effect of MDA-MB-231 treated with 4ab at concentrations 0, 1, 2, 4, 8, and 16 µM. (C, D) represent the effect of 4ab on autophagy-related protein. MDA-MB-231 cells were treated with 4ab with different concentrations; the expression of LC3-II and p62 were analyzed by western blotting. Data represent the mean±SD *p<0.05, **p<0.01, ***p<0.001.
**Figure 2**

4ab inhibits PI3K/AKT/mTORC1 pathway. Western blot representation and quantitative expression of p-PI3K, p-AKT, and p-mTORC1 in MDA-MB-231 cells treated with 4ab at various concentrations in attached (A,B) and detached (C,D) conditions. Data represent the mean±SD *p<0.05, **p<0.01, ***p<0.001

**Figure 3**
4ab induces sustained UPR induction in aggressive tumor cells. (A) Morphological images of TNBCs cells upon 4ab treatment on 0, 3, 12, 24 h; 16 µM. (B, C) Western analysis of 4ab treated MDA-MB-231 cells with concentrations 0, 1, 2, 4, 8, 16 µM; 24 h. (D, E) immunoblotting analysis of MDA-MB-231 cells timely (0, 3, 6, 12 and 24 h) treated with 4ab (16 µM) in floating condition. Relative protein expression intensity was measured by using Software Image J. Data represent the mean±S.D.; *p<0.05, **p<0.01, ***p<0.001 vs control.

Figure 4

Intrinsic apoptotic pathway activated by 4ab in MDA-MB-231 cells. (A, B) TMRE fluorescence (red) intensity was detected and measured by fluorescence microscopy in MDA-MB-231 cells treated with 4ab. (C, D) immunoblot representation of p-JNK, Bax, Cytochrome c, Caspase-3 and PARP-1 in the whole cell lysate of MDA-MB-231 treated with 0, 1, 2, 4, 8 and 16 µM of 4ab for 24 h and their quantification. For data analysis Image J software was used. Data represent the mean±SD **p<0.01, ***p<0.001
Figure 5

4ab is responsible for pancreatic adenocarcinoma cell death through activating JNK- autophagy-apoptosis axis (A) Morphological effect on MIA-PaCa-2 cells after treatment with 4ab with 0, 0.125, 0.25, 0.5, 1, 2 µM for 24, yellow circles represent vacuolation in cells. (B, C) Effect of 4ab on the expression of JNK, autophagy marker (LC3-II, p62) and apoptosis mediators (Bax, caspase-3, and PARP-1) in MIA PaCa-2 cells by western blotting analysis. The intensity of these proteins was quantified by using Image J software. Data represent the mean±SD *p<0.05, **p<0.01, ***p<0.001
Figure 6

4ab inhibits the aggressiveness of TNBCs. (A, B) Represents images of MDA-MB-231 cells in Boyden chamber (pore size 8 μm) treated with different concentrations (0, 1, 2, 4, 8 and 16 μM) of 4ab for 24 h. (C, D) showing colony formation potential of MDA-MB-231 cells after 24 h treatment of 4ab. (E, F) Figure demarcates the effect on migration potential of MDA-MB-231 when treated with 0, 1, 2, 4, 8, 16 μM of 4ab at 0, 12, 48 h. For data analysis, ImageJ software was used. Data represent the mean±SD **p<0.01, ***p<0.001
Figure 7

**Non-aggressive cancer cells were resistant to the effect of 4ab** (A) Morphological image representation of 4ab treated MCF-7 cells for 24 h in a concentration-dependent manner. (B) Effect of 4ab on protein expression-p-JNK, p-AKT, Caspase-3 and Parp-1 in MCF-7 cells.
**Figure 8**

4ab reduced the 4T1 metastatic burden in BALB/C. (A) Image representation of 4T1 (1×10^6 cells/mouse) injected in BALB/C mice with grouping (6 mice each group) vehicle, control, 4ab 5mg/kg and 4ab 10 mg/kg on 31-day post injection (p.o). and lung metastasis development in different groups. (B, C) Protein expression and quantification of tumors from desired mice groups. Data represent the mean of three biological replicates and mean±SD **p<0.01, ***p<0.001

**Supplementary Files**

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