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Misregulation of BCL-2 family of proteins renders a survival signal to withstand cytotoxic anticancer drugs and is often found in drug resistant cells. The drug resistance phenotype is also associated with an enhancement of cancer stem cell-like (CSC) characteristics. Thus, inhibition of anti-apoptotic BCL-2 family proteins has been proposed as a possible antineoplastic strategy, and BCL-2 inhibitors are currently being clinically trailed in patients with leukemia, lymphoma or non-small cell lung cancer. However, the effects of BCL-2 inhibitors on drug resistant breast cancer have not yet been elucidated. In the present study, the effect of sabutoclax, a pan-active BCL-2 protein family antagonist, on two chemoresistant breast cancer cell lines was assessed. We found that sabutoclax showed a significant cytotoxic activity on chemoresistant breast cancer cells both *in vitro* and *in vivo*. When chemotherapeutic agents were combined with sabutoclax, strong synergistic antiproliferative effects were observed. Sabutoclax induced the blockage of BCL-2, MCL-1, BCL-xL and BFL-1, which in turn led to caspase-3/7 and caspase-9 activation and modulation of Bax, Bim, PUMA and survivin expression. Furthermore, sabutoclax effectively eliminated the CSC subpopulation and reduced sphere formation of drug-resistant cells through down-regulation of the IL-6/STAT3 signaling pathway. A similar effect was observed in a small panel of nine breast tumors *ex vivo.* Our findings indicate that sabutoclax partially overcomes the drug resistance phenotype of breast cancer cells by reactivation of apoptosis, mediated by the inhibition of several anti-apoptotic BCL-2 family proteins, and eliminates CSCs by abolition of the IL-6/STAT3 pathway. This offers a strong rationale to explore the therapeutic strategy of using sabutoclax alone or in combination for chemotherapy-nonresponsive breast cancer patients.

Sabutoclax, pan-active BCL-2 protein family antagonist, overcomes drug resistance and eliminates cancer stem cells in breast cancer

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Abstract

Misregulation of BCL-2 family of proteins renders a survival signal to withstand cytotoxic anticancer drugs and is often found in drug resistant cells. The drug resistance phenotype is also associated with an enhancement of cancer stem cell-like (CSC) characteristics. Thus, inhibition of anti-apoptotic BCL-2 family proteins has been proposed as a possible antineoplastic strategy, and BCL-2 inhibitors are currently being clinically trailed in patients with leukemia, lymphoma or non-small cell lung cancer. However, the effects of BCL-2 inhibitors on drug resistant breast cancer have not yet been elucidated. In the present study, the effect of sabutoclax, a pan-active BCL-2 protein family antagonist, on two chemoresistant breast cancer cell lines was assessed. We found that sabutoclax showed a significant cytotoxic activity on chemoresistant breast cancer cells both *in vitro* and *in vivo*. When chemotherapeutic agents were combined with sabutoclax, strong synergistic antiproliferative effects were observed. Sabutoclax induced the blockage of BCL-2, MCL-1, BCL-xL and BFL-1, which in turn led to caspase-3/7 and caspase-9 activation and modulation of Bax, Bim, PUMA and survivin expression. Furthermore, sabutoclax effectively eliminated the CSC subpopulation and reduced sphere formation of drug-resistant cells through down-regulation of the IL-6/STAT3 signaling pathway. A similar effect was observed in a small panel of nine breast tumors *ex vivo.* Our findings indicate that sabutoclax partially overcomes the drug resistance phenotype of breast cancer cells by reactivation of apoptosis, mediated by the inhibition of several anti-apoptotic BCL-2 family proteins, and eliminates CSCs by abolition of the IL-6/STAT3 pathway. This offers a strong rationale to explore the therapeutic strategy of using sabutoclax alone or in combination for chemotherapy-nonresponsive breast cancer patients.

1. Introduction

Breast cancer still remains the most frequent malignancy among women worldwide [1]. Despite improvements in detection and treatment options leading to excellent short-term prognoses, there is a high percentage of non-responders to chemotherapy after relapse. This represents a major challenge for the management of breast cancer patients, especially in the metastatic setting [2; 3; 4]. Understanding the mechanisms by which chemotherapy resistance develops is of the outmost importance for the development of novel therapeutic strategies.

Growing evidence indicates that a subset of cancer cells, with self-renewal and differentiation properties, termed cancer stem-like cells (CSCs), are resistant to chemotherapy [5; 6]. Breast cancer stem cells (BCSCs) have been demonstrated to possess specific functional properties such as elevated aldehyde dehydrogenase (ALDH) activity, robust DNA damage repair, over-expression of ABC transporters and abnormal activation of numerous signaling pathways, such as Notch, PI3K/AKT and IL-6/STAT3 pathways [7; 8; 9]. Furthermore, an enrinchment of BCSCs has been demonstrated in breast cancer patients after neoadjuvant chemotherapy, providing mechanistic clues to chemotherapeutic resistance [10; 11]. Moreover, an increase in the BCSC population is associated with shorter cumulative disease-free survival and overall survival of invasive breast carcinoma patients [12; 13]. Taken together, these studies suggest that drugs selectively targeting BCSCs should improve the outcome of breast cancer patients, particularly in combination with chemotherapy.

Impairment of apoptosis is a hallmark of cancer [14] and deregulated expression of BCL-2 family of proteins is a key mechanism leading to apoptosis evasion and drug resistance [15]. In addition to clinical prognostic markers in breast cancer, BCL-2, BCL-XL and MCL-1 are over-expressed in stem cell-like cancer cells [16; 17; 18; 19]. We have previously shown that over-expression of miR-218 or EP300 increases chemotherapy sensitivity in breast cancer cells by modulation of the BCL-2 family of proteins [20]. Consequently, we hypothesized that small molecule inhibitors of BCL-2 family proteins might be an effective therapeutic strategy to sensitize BCSCs.

Sabutoclax (BI-97C1) is a novel Apogossypol derivative BCL-2 homology domain 3 (BH3) mimetic, with pan-BCL-2-inhibitory potency [21; 22]. This compound binds to BCL-2, MCL-1, BCL-XL, BFL-1 and exerts strong efficacy against leukemia [23], prostate cancer [24], colorectal cancer [25] and pancreatic cancer [26]. Several recent reports indicate enhanced antitumor effects in mouse models when sabutoclax was co-administered with chemotherapeutic agents or inhibitors of other signaling pathways [23; 26; 27].

In this study, we analyzed the efficacy of sabutoclax on two chemoresistant breast cancer cell models. Sabutoclax exerted potent efficacy triggering apoptosis as well as eliminating BCSCs *in vitro*, *ex vivo* and *in vivo*. It also down-regulated IL-6/STAT3 signaling, a key pathway mediating breast cancer chemoresistance and BCSCs properties. In addition, sabutoclax synergized *in vivo* with doxorubicin, a common chemotherapeutic agent of breast cancer, and was well tolerated. These results suggest that sabutoclax in combination with standard chemotherapeutic drugs targets BCSCs and resensitizes chemotherapy resistant breast cancer.

2. Materials and Methods

2.1 Cells and drugs

Human breast cancer cell line MCF-7 and its multidrug resistant (MDR) derivative MCF-7/A02 were gifts from Professor Dongsheng Xiong (Institute of Hematology, PUMC, Tianjin, China) and were cultured as previously described [20]. Human breast cancer cell line Cal51 and its MDR derivative CALDOX were cultured as previously described [28]. Doxorubicin, etoposide and taxol were from Sigma (St Louis, MO, USA); Sabutoclax, ABT-737, z-VAD-fmk and WP1066 were from Selleck (Houston, TX,USA); IL-6 was from R&D System (Oxon, UK).

2.2 Cell viability analysis

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed to evaluate the cell viability in response to drug treatments and were also used to determine the concentration of drug that inhibited cell growth by 50% (IC_{50}) after 3 days of treatment. For drug combination experiments, a combination index (CI) number was calculated using the CalcuSyn software (Biosoft, Cambridge, UK) based on the Chou and Talalay method [29]. CI values between 0.1 and 0.9 define different grades of synergism: values between 0.9 and 1.1 are additive, whereas values $>$ 1.1 are antagonistic.

2.3 Drug resistance clonogenic assay

Cells at a density of 2×10^5 cells/well in 6-well plates were treated with a single dose of doxorubicin (3 μM for MCF-7 and MCF-7/A02 cells; 0.2 μM for Cal51 and CALDOX cells), or 5 μM sabutoclax for 1 week. Resistant clones were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet and counted. Crystal violet retained in the cells was solubilized with 0.5% acetic acid and quantified by measurement of optical density at 592 nm.

2.4 Annexin V staining

Cell apoptosis was measured by flow cytometry using an annexin V-FITC apoptosis detection kit (Becton-Dickinson, San Diego, CA, USA) essentially as described [20].

2.5 Caspase activity

Cells (1×10^4) were incubated with Sabutoclax or ABT-737 in a 96-well plate for 24h. Caspase-3/7 and caspase-9 activities were measured using Caspase-Glo 3/7 Assay and Caspase-Glo 9 Assay Kits, respectively (Promega, Madison, WI, USA) following the protocol recommended by the manufacturer. Z-VAD-fmk was used as a pan-caspase inhibitor [28].

2.6 RNA isolation and reverse transcription quantitative real-time–PCR (RT-qPCR)

RNA isolation, reverse transcription and real-time qPCR were performed as previously described [20] using specific primers for each gene (Supplementary Table S3). Relative transcript levels were normalized to the expression of *RPS14* mRNA.

2.7 Antibodies

Antibodies for immunodetection following immunoblotting procedures [28] were BAX (D2E11), cleaved PARP (D64E10), cleaved caspase 3 (5A1E), MCL-1 (D35A5), BCL-XL (54H6), BIM (C34C5), PUMA (D30C10) (Cell Signalling Technology), Survivin (ab76424, Abcam, Cambridge, UK), pSTAT3 (Tyr705)(YP2051), STAT3 (YM0597), BCL-2 (YM3041) and β-actin (YM1207) (Immunoway, Plano, TX, USA). Appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology) were detected by enhanced chemiluminiscence (Millipore, Billerica, MA, USA) [28].

2.8 Enzyme-linked immunosorbent assay (ELISA)

Cells (3×10^5) were plated in a six-well plate with Dulbecco's Modified Eagle Medium (DMEM, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS) and incubated for 24 h.

Medium was replaced with 2 ml serum-free DMEM and collected for ELISA 12 h after sabutoclax treatment. Blood was taken from tumor-bearing mice and immediately placed into EDTA-treated tubes (BD Biosciences, San Jose, CA, USA). Plasma was obtained by removing blood cells through centrifugation at 1800 × g for 15 min. IL-6 levels in both the cell culture medium and plasma of mice were measured by commercial IL-6 ELISA Kit (R&D Systems, Oxon, UK) according to the manufacturer's instructions. This kit uses antibodies to recognize human IL-6 with no cross-reactivity with the mouse corresponding proteins.

2.9 Immunohistochemistry (IHC)

Clinical data from 9 breast cancer patients obtained from Tianjin Medical University Cancer Institute and Hospital from June 2016 to Jun 2017 are presented in Table 2. All patients provided written informed consent for research use and the research was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Untreated primary tumor samples from all of the patients were collected by core needle biopsy following standard clinical procedures. Within 1h post-biopsy, samples were washed with sterile PBS several times and put into cell culture flasks containing DMEM supplemented with 10% FBS. When indicated, sabutoclax (30 μM) was added to the medium and the culture flasks kept for 48 h at 37℃ in a 5% CO_2-air atmosphere. After *ex vivo* culture and drug treatment, tumor samples were fixed in formalin, embedded in paraffin and sectioned for immunohistochemistry staining by using anti-cleaved caspase 3, survivin, BAX, CD44, ALDH1 and pSTAT3 (Tyr705) antibodies as previously described [30].

The staining score was blindly evaluated by two pathologists using the following staining scoring criteria: 0, 1%-4% of the cells stained positive; 1, 5%-25% of the cells stained positive; 2, 26%-50% of the cells stained positive; 3, 51%-75% of the cells stained positive; and 4, more than 76% of the cells stained positive. The staining intensity of cancer cells was scored as 0 (no staining), 1 (weak staining), 2 (intermediate staining), and 3 (strong staining). The multiple of the intensity and extent score was used as the final score (0-12) for the expression of each protein [31].

2.10 Soft agar colony formation assay

Cells were dissociated with trypsin following standard cell culture procedures and suspended in DMEM containing 0.3% agar and plated on the top of a solidified layer of 0.6% agar in DMEM The cells were plated at a density of 2×10^5 cells/well in six-well plates, and the colonies were counted 21 days later after staining with MTT [28].

2.11 Mammosphere culture

Cells (1×10^3) were plated in each well of an ultralow attachment plate (Corning, NY, USA) with 3 ml serum-free mammary epithelial growth medium (MEGM, BioWhittaker, Walkersville, MD, USA), supplemented with B27 (Invitrogen), 20 ng/ml EGF and 20 ng/ml basic FGF (BD Biosciences). Sphere formation was assessed 10 days later [28].

***2.12 In vivo* xenografts**

Cells (1×10^7) were suspended in 100 μl PBS containing 50% Matrigel (BD Biosciences) and injected into the mammary fat pad of 4-5 week old female nude mice (Vital River Company, Beijing, China). Tumor size was measured every 3 days in two dimensions using a caliper, and the tumor volume was calculated with the following formula: tumor volume (mm^3) $=0.5 \times ab^2$ (a and b being the longest and shortest diameters of the tumor, respectively). Five days after cell injection, the tumor-bearing mice were randomly divided into four groups (five animals/group). Mice were

treated with PBS, 2 mg/kg DOX, 5 mg/kg sabutoclax, or both DOX and sabutoclax at the same doses. Sabutoclax was dissolved in a 10:10:80 solution of ethanol: Cremophor: PBS. DOX was dissolved in PBS. Mice were treated with DOX, sabutoclax or both drugs every 3 days via i.p. injection for a total of 6 injections. Twenty-four hours after the final drug treatment, mice were killed in a humane manner and tumors collected for further investigations, including RNA extraction and paraffin embedding for IHC staining and TUNEL assays. For *in situ* apoptosis detection in tumor xenograft sections, TUNEL reaction was performed using the In situ Cell Death Detection kit (Roche Molecular Biochemicals, Indianapolis, USA) according to the manufacturer's instructions. All animal studies were performed at the National Institutes of Health (Tianjin Cancer Hospital) in accordance with guidelines under Institutional Animal Care and Use Committee (IACUC) and approved by the Committee on the Ethics of Animal Experiments of the Tianjin Cancer Hospital.

2.13 Statistical analysis

Comparisons of the means among more than two groups were performed by one-way analysis of variance. Student's *t*-test was used when comparing the means of two groups. A *P*-value<0.05 was considered statistically significant.

3. Results

3.1 Sabutoclax shows potent cytotoxicity against both sensitive and chemoresistant breast cancer cell lines

MCF-7/A02 and CALDOX cells, that were derived from chemosensitive MCF-7 and Cal51 cell lines, respectively, show a traditional chemoresistant phenotype with cross-resistance to a wide range of structurally and functionally unrelated drugs (supplementary Tables S1 and S2). Increased anti-apoptotic and/or decreased pro-apoptotic BCL-2 proteins expression has been implicated in the development of resistance to standard therapies in breast cancer [32; 33; 34]. Although the mechanisms of chemoresistance in MCF-7/A02 and CALDOX cells are quite different [35; 36], we found higher levels of BCL-2, MCL-1 and BCL-XL, as well as lower levels of BIM, BAX and PUMA (Figure 1A). Thus, we hypothesized that targeting anti-apoptotic BCL-2 proteins could be an effective way of resensitising chemoresistant breast cancer cells. As expected, sabutoclax inhibited cell growth of both chemosensitive and chemoresistant breast cancer cells in a dose-dependent manner (Supplementary Figure S1A). Drug sensitivity curves indicated that resistance to sabutoclax was modest in both MCF-7/A02 and CALDOX cells (3.46- and 1.65-fold, respectively; Figure 1B). Importantly, non-tumorigenic MCF-10A cells showed very little growth inhibition, with an IC_{50} value greater than 40 µM in contrast to ~1.5 µM and 5 µM in MCF-7 and Cal51 cells, respectively (Supplementary Figure S1B). This is in agreement with a recent report indicating that non-tumorigenic pancreatic cells respond poorly to sabutoclax treatment [26]. These effects were validated further using clonogenic assays. Sabutoclax significantly decreased the capacity of chemoresistant cells to survive after one week treatment (reduction ranging from 52% to 68%, Figure 1C and Supplementary Figure S1C). Thus, these results demonstrate that sabutoclax is a potent cytotoxic agent to chemoresistant breast cancer cells.

3.2 Sabutoclax promotes cell apoptosis, induces caspase activity and modulates apoptosis-related proteins

As sabutoclax is a pan-BCL-2 family inhibitor, we asked whether its cytotoxic effect on chemoresistant breast cancer cells is due to activation of apoptosis. For these assays we used etoposide as a positive control, another widely used chemotherapeutic drug known to induce cell apoptosis [37; 38], as DOX fluorescence interferes with annexin V/propidium iodide (PI) staining [39]. As expected, 20 µM etoposide induced apoptosis in MCF-7 cells, but failed to do so in MCF-7/A02 cells (Figure 1D). However, 5 µM sabutoclax was able to induce apoptosis both in MCF-7 and MCF-7/A02 cells. A similar effect was also observed in CALDOX cells (Figure 1D). In agreement with the annexin V/PI analyses, sabutoclax strongly induced caspase-3/7 and caspase-9 activities in a dose-dependent manner, in both MCF-7/A02 and CALDOX cells (Figure 1E). Caspase activation was reduced by the pan-caspase inhibitor z-VAD-fmk (Supplementary Figure S1D), resulting in IC_{50} shifts of 5.48-fold in MCF-7/A02 and 3.39-fold in CALDOX cells (Supplementary Figure S1E and F). These data demonstrate the specific induction of cell death by sabutoclax through activation of the apoptotic cascade.

Caspase 3, the effector caspase in the apoptotic cascade, is activated through cleavage by caspase-9 as a result of increased mitochondrial permeability and release of cytochrome c. PARP is cleaved by activated caspase-3 and cleavage of caspase-3 and PARP are signature events in apoptosis [40]. Consistent with the apoptosis induced by sabutoclax, we observed a marked increase in the levels of both cleaved caspase-3 and PARP in CALDOX cells after sabutoclax

treatment (Figure 1G). MCF-7 cells lack caspase 3 [41] and these activities were not measured in MCF-7/A02 cells. As sabutoclax binds to BCL-2, MCL-1, BCL-XL, BFL-1 and the induction of apoptotic cell death could be due to alteration of apoptosis-related genes, we also evaluated the mRNA levels of both survival and apoptotic genes. Reverse transcription quantitative real-time-PCR (RT-qPCR) and western blot results revealed that sabutoclax repressed expression of pro-survival gene Survivin and upregulated pro-apoptotic genes BIM, PUMA and BAX in chemoresistant cells (Figure 1F&G).

To further confirm that the promoting effect of sabutoclax on apoptosis is specifically mediated by pan-BCL-2-inhibition, chemoresistant breast cancer cells were treated with ABT-737, another well-characterized BH3 mimetic with high affinity for BCL-2, BCL-xL and BCL-w. Similar to sabutoclax, the IC_{50} values of ABT-737 in MCF-7/A02 and CALDOX cells were 13.3 and 4.3 times greater than those in MCF-7 and Cal51 cells, respectively (Figure S2A). ABT-737 also induced cell apoptosis and activated caspases in both MCF-7/A02 and CALDOX cells (Figure S2B and C). In addition, ABT-737 treatment increased the levels of cleaved caspase-3 and PARP in CALDOX cells and enhanced BAX and BIM expression at both mRNA and protein levels (Figure S2D and E). However, ABT-737 displayed weaker growth inhibitory and apoptosis induction than sabutoclax. This is not surprising since ABT-737 lacks of affinity for MCL-1, which is commonly over-expressed in breast cancer [42; 43; 44]. Thus, inhibition of BCL-2 families with sabutoclax offers a promising strategy for the reactivation of apoptotic pathways in chemoresistant breast cancer.

3.3 Sabutoclax eliminates the stem cell subpopulation of chemoresistant breast cancer cells

CSCs are thought to play a very important role in the generation of cell populations resistant to therapy, due to increased availability of drug protective mechanisms [45]. As sabutoclax showed robust anticancer properties in chemoresistant breast cancer cells, we asked whether it could also eliminate the stem cell-like subpopulation from these cells. First, we tested the effect of sabutoclax on the proportion of stem-like cells (SCs) in chemoresistant breast cancer cell population (total cells, TCs). We analyzed the expression of CD44 and CD24 by flow cytometry, as $CD44^{high}/CD24^{low}$ cells are associated with BCSCs [45]. As expected, chemoresistance was accompanied by an increase (40%) in the $CD44^{high}/CD24^{low}$ subpopulation in MCF-7 cells, that was reduced in a dose dependant manner after sabutoclax treatment (Figure 2A) . As Cal51 and CALDOX cells have a very low CD44 expression [46], we then analyzed aldehyde dehydrogenase (ALDH) activity, another important marker of SCs [28]. As expected in a basal-like triple negative cell line, Cal51 cells showed a high proportion of ALDH-positive cells (~35%), that was increased further to 55% in CALDOX resistant cells. Importantly, sabutoclax reduced this CALDOX subpopulation in a dose-dependant manner, reaching levels comparable to those found in naïve Cal51 cells when used at 5 µM, and lower than those when used at 10 µM. Naïve luminal MCF-7 cells showed, as expected, a very small subpopulation of ALDH-positive cells (~5%) that increased to 55% in drug resistant MCF-7/A02 cells. In line with the effects of sabutoclax on the $CD44^{high}/CD24^{low}$ subpopulation, sabutaclax also decreased the percentage of ALDH-positive MCF-7/A02 in a dose-dependent manner and, when used at 15 µM, down to the levels found in naïve MCF-7 cells (Figure 2B).

BCSCs exhibit other stem-like properties, including an increase in anchorage independence, that can be determined by their ability to survive and grow as spheres or colonies in low attachment plates and soft agar medium, respectively. In order to test the effect of sabutoclax on

these BCSC properties, we first confirmed that both MCF-7/A02 and CALDOX cells have increased anchorage independence. There was higher proportion of spheres and colonies in drug resistant deriviatives than in naïve cells. MCF-7/A02 showed increases of 104% and 22%, whereas CALDOX showed increases of 65% and 86% in spheres and colonies, respectively. Importantly, sabutoclax treatment practically abolished MCF-7/A02 and CALDOX cells sphere forming efficacy, as well as their ability to generate colonies in soft agar (Figure 2C&D and Supplementary Figure S3A). It is also notable that after sabutoclax treatment the large spheres completely disappeared, and most of the surviving cells undertook apoptosis (Figure 2C), making them too fragile to be enzymatically dispersed to single cells and assayed for their ability to form secondary mammospheres. Thus, sabutoclax eliminates the SCs subpopulation and inhibits SCs survival *in vitro*.

Growth of mammospheres is a well-established technique for the enrichment of highly tumorigenic SCs [28], and we obtained mammosphere cells (MCs) from MCF-7/A02 and CALDOX cells after growth in low attachment flasks with the aim of assessing sabutoclax effects. MCs showed an increase in those characteristics associated with BCSCs, including a larger $CD44^{high}/CD24^{low}$ subpopulation and higher proportion of ALDH-positive cells (Supplementary Figure S3 B&C) than TCs. Drug sensitivity assays indicated that DOX and etoposide were more cytotoxic in chemoresistant TCs than in their MCs. MCF-7/A02 and CALDOX MCs exhibited an increase in DOX resistance of 68.8- and 47.6-fold, respectively, when compared to their TCs (Table 1). Similarly, the increase in etoposide resistance was 42.3- and 18.2-fold in MCF-7/A02 and CALDOX MCs, respectively (Table 1). Sabutoclax also displayed a cytotoxic effect on MCs isolated from MCF-7/A02 (IC_{50} 48.9 μM) and CALDOX (IC_{50} 34.8 μM) (Table 1 & Figure 2E), as well as dose-dependent caspase activation (Figure 2F). Compared with TCs, the resistance of MCs to sabutoclax ranged from 3.61- to 6.38-fold (Table 1), indicating that this pan-BCL2 inhibitor is more toxic to MCs than DOX and etoposide.

3.4 Sabutoclax eliminates the stem cell subpopulation through down-regulation of the IL-6/STAT3 signaling pathway

The IL-6/STAT3 pathway is constitutively activated in BCSCs and contributes to their proliferation, stemness and chemoresistance [47; 48; 49]. Bcl-2 expression positively correlates with IL-6/STAT3 activation in several solid tumors, including breast cancer [33; 50; 51], and sabutoclax treatment results in dephosphorylation of STAT3 at Tyr705 in pancreatic cancer [26]. Therefore, we speculated that sabutoclax could affect the activation of the IL-6/STAT3 pathway in chemoresistant breast cancer cells leading to SCs elimination. To address this issue, we firstly confirmed IL-6 over-expression and STAT3 phosphorylation in both MCF-7/A02 and CALDOX cells (Figure 3A&B). Then, we examined mRNA and protein levels of IL-6, as well as STAT3 phosphorylation levels, in MCF-7/A02 and CALDOX cells before and after sabutoclax treatment. The results showed that sabutoclax significantly decreased IL-6 expression and STAT3 phosphorylation (Figure 3C&D).

To further verify whether the SCs elimination observed after sabutoclax treatment was due to down-regulation of IL-6/STAT3 activation, a rescue methodology was adopted. For this, cells, untreated or pretreated with IL-6 for 2h, were treated with sabutoclax and analyzed for STAT3 phosphorylation by western blot and the proportion of SCs ($CD44^{high}/CD24^{low}$ population and ALDH activity) by flow cytometry. The addition of IL-6 restored STAT3 phophorylation in

MCF-7/A02 and CALDOX cells treated with sabutoclax (Figure 4E). In addition, an increase (~10 %) of $CD44^{high}/CD24^{low}$ cells was found in sabutoclax-treated MCF-7/A02 cells upon IL-6 addition (Figure 3F). Similarly, the addition of IL-6 to sabutoclax-treated MCF-7/A02 and CALDOX cells increased the percentage of ALDH-positive cells (Figure 3G, Supplementary Figure S3E).

In order to confirm that STAT3 activity is involved in the sabutoclax/IL-6 axis regulating the SCs population and stemness of breast cancer cells, MCF-7/A02 and CALDOX cells were treated with WP1066, a well-characterized STAT3 specific inhibitor [52]. Blocking STAT3 activity caused a decrease in STAT3 phosphorylation that was accompanied by a reduction in the SCs populations of MCF-7/A02 and CALDOX cells (Figure 3H&I, Supplementary Figure S3E).

Overall, the toxic effect of sabutoclax on SCs from chemoresistant breast cancer cells is due to IL-6 down-regulation, and can be mimicked by suppression of STAT3 activity.

3.5 Sabutoclax treatment induces apoptosis and reduces SCs population in human breast tumors

In order to validate the results showed in breast cancer cell lines, we tested the *ex vivo* effect of sabutoclax on nine fresh human breast tumor samples (Table 2) by IHC. Quantitation of cleaved-caspase3 staining indicated the presence of significant apoptosis of breast epithelia in most of sabutoclax-treated tumor samples (median values of 5.67 in the control versus 8 in the sabutoclax-treated group, $p<0.05$). In agreement with the activation of apoptosis, lower expression of survivin and higher expression of BAX were also observed in sabutoclax-treated tumor samples. Moreover, stronger CD44 and ALDH1 staining were observed in the control than in the sabutoclax-treated group, indicating an elimination of SCs in breast tumors by sabutoclax. In addition, sabutoclax-treated tumors showed decreased STAT3 phophorylation (Fig 4A). It is worthy of note that 2 of the 9 tumor samples were from residual tumor tissue of the patients after receiving neoadjuvant chemotherapy treatment. Both patients received a TAC regimen of doxorubicin (50 mg/m^2, q21d), docetaxol (75 mg/m^2, q21d) and cyclophosphamide (500 mg/m^2, q21d) on day 1. Treatment was continued in the absence of unacceptable toxicity for six cycles given every 21 days. In patients lacking pathologic complete response, neoadjuvant chemotherapy selects a subpopulation of chemotherapy-resistant tumor cells [32; 53]. IHC results of the tumor samples from these two patients further demonstrated that sabutoclax treatment effectively induced apoptosis and reduced SCs population in chemoresistant tumor tissues (Fig 4B). Altogether, these results on *ex vivo* tumor samples provide further support to the role of sabutoclax triggering apoptosis and SCs elimination.

3.6 Sabutoclax acts synergistically with chemotherapeutic agents both *in vitro* and *in vivo*

The efficacy of chemotherapy, endocrine-based therapies and targeted drugs is often thwarted by increased anti-apoptotic and /or decreased pro-apoptotic BCL-2 proteins. Recent studies indicate that targeting BCL-2 in combination with trastuzumab is beneficial in trastuzumab-resistant breast cancer [54], and combination therapy of a BH3 mimetic with chemotherapy or mTOR inhibitors induces a synergistic tumor response [55; 56]. Hence, we further investigated whether sabutoclax could synergize with chemotherapeutic agents in breast cancer therapy, especially for treating chemoresistant breast cancer. For this purpose, MCF-7/A02 and CALDOX cells were treated with increasing concentrations of DOX/etoposide, either alone or in combination with sabutoclax at fixed ratios (DOX/sabutoclax, 16:5 for MCF-7/A02; etoposide/sabutoclax, 10:1 for

MCF-7/A02; DOX/sabutoclax, 2:5 for CALDOX; etoposide/sabutoclax, 8:5 for CALDOX). The combination index (CI) values ranged from 0.248 to 0.735, indicating that DOX, or etoposide, and sabutoclax used in combination acted synergistically in both MCF-7/A02 and CALDOX cells (Table 3). In addition, the Annexin V/PI analysis showed that the combination treatment of sabutoclax and etoposide increased cell apoptosis to a higher extent than each drug alone, in both MCF-7/A02 and CALDOX cells (Figure 5A). Colony forming assays further confirmed that the combination treatment of sabutoclax and DOX inhibited the formation of resistant clones more efficiently than each drug alone, in both MCF-7/A02 cells (DOX+sabutoclax: 92.9% vs DOX: 10.1% and sabutoclax: 57.8%) and CALDOX cells (DOX+sabutoclax: 84.4% vs DOX: 8.5% and sabutoclax: 43.2%) (Figure 5B).

To further investigate whether the synergistic effect of sabutoclax could be maintained *in vivo*, chemoresistant MCF-7/A02 cells were injected into the mammary fat pad of female nude mice. On day 5 after injection, mice were randomly divided into four groups with equal number of mice. Each group was treated with sabutoclax, DOX, sabutoclax plus DOX or vehicle control. As expected, DOX treatment alone showed minimal effects, whereas sabutoclax alone showed a significant reduction in tumor growth (Figure 5C). Moreover, animals treated with a combination of sabutoclax and DOX showed a complete absence of tumor growth. Notably, single sabutoclax therapy was well tolerated, as mice maintained normal body weight (BW) during the 20 days of treatment. In contrast, the animals receiving DOX alone displayed a significant BW loss (~25.4% reduction by day 20 as compared with their BW on day 5). These results suggest that combining sabutoclax with chemotherapy has the potential to enhance tumor response without a further increase in toxicity.

Consistent with the *in vitro* results, combination of sabutoclax with DOX increased both TUNEL signal and cleaved-caspase 3 expression with respect to single agent treatments (Figure 5D). In addition, IHC of sabutoclax-treated tumors showed significantly less phosphorylated STAT3 expression, CD44 expression and ALDH1 expression than control group tumors (Figure 5E). Sabutoclax-treated tumors also showed lower human IL-6 mRNA levels, and this was further confirmed at the protein level when human IL-6 plasma concentrations were determined (Figure 5F). Altogether, these data strongly indicate that sabutoclax effectively blocks the activity of the IL-6/STAT3 pathway inducing cell apoptosis and diminishing SCs population. This subsequently reduces tumor growth *in vivo*, which is practically abolished with the addition of a chemotherapeutic agent.

4. Discussion

Resistance to chemotherapeutic drug treatment in breast cancer patients still remains one of the biggest clinical challenges. Chemoresistance is a complex phenomenon involving multiple mechanisms. As several anti-apoptotic BCL-2 proteins are frequently up-regulated in chemoresistant breast cancer cells, we initially evaluated the efficacy of sabutoclax, a BH3 mimetic that targets these anti-apoptotic proteins and inhibits their function. Sabutoclax was effective as a single agent in both chemo-sensitive and chemoresistant breast cancer cell lines, while it had little cytotoxic effect in a non-tumorigenic breast cell line. Others have also reported that sabutoclax inhibits cell growth in multiple, genetically diverse, pancreatic cancer cell lines, but has no effect on a normal cell line [26], and cytotoxic activity against B-cell lymphoma and leukemia cells with little hepatotoxicity and gastrointestinal toxicity in mice [57]. Our results also indicate that sabutoclax can augment the cytotoxic activity of traditional chemotherapies without an increase in toxicity in the nude mice. Although no current clinical trials are addressing the effect of sabutoclax, or other inhibitors of anti-apoptotic BCL-2 family members, in breast cancer, initial Phase I studies in small cell lung cancers suggest that ABT-263 (a BCL-2/BCL-xL/BCL-w inhibitor) is safe and well tolerated in patients with solid tumors [58]. Interestingly, BH3 mimetics alone, including ABT-737, ABT-199, ABT-263 and S63845, are insufficient to inhibit the growth of breast cancer in xenograft or PDX models [59; 60]. However, our results, as well as those from others, indicate that sabutoclax alone attenuates tumor growth in *in vivo* assays of breast, prostate and pancreatic cancer, probably due to MCL-1 targeting [26; 27]. These preclinical results provide a rationale for the development of clinical protocols to evaluate sabutoclax in breast cancer patients.

Sabutoclax is more effective reducing chemoresistant breast cancer cell viability than traditional chemotherapeutic agents such as doxorubicin and etoposide. It is well established that therapy resistance can, in many cases, be partially overcome by inhibiting anti-apoptotic BCL-2 family proteins. For instance, ABT-737 increases sensitivity to trastuzumab in trastuzumab-resistant breast cancer cells [54], and experimental down-regulation of BCL-2 using a histone deacetylase inhibitor restores sensitivity to tamoxifen in hormone therapy-resistant breast cancer cells [61]. Sabutoclax also sensitizes human prostate cancer cells to mda-7/IL-24 by inhibition of MCL-1 [24]. Here we show that inhibition of anti-apoptotic BCL-2 family proteins by ABT-737 leads to apoptosis of chemoresistant breast cancer cells. However, MCL-1 is an important mediator of apoptotic escape and therapeutic resistance in breast cancer [62; 63]. As ABT-737 has low affinity for MCL-1, ABT-737 is less cytotoxic than sabutoclax in chemoresistant breast cancer cells. Additionally, MCL-1 limits the efficacy of ABT-263 and ABT-737, as other MCL-1 inhibitors show strong synergistic cytotoxicity when combined with either of these two drugs in breast cancer [64; 65]. Thus, there is a strong mechanistic rationale for the enhanced therapeutic efficacy of sabutoclax in breast cancer cells by inhibition of BCL-2, MCL-1, BCL-XL and BFL-1.

Despite the potential of sabutoclax as a single agent, there is a pressing need for combination therapies in the clinical setting, as resistance arises more frequently to single drugs than to those used in combination. Here we show that sabutoclax exerts a synergistic effect with doxorubicin both *in vitro* and *in vivo*. Others have shown that sabutoclax renders human leukemia stem cells sensitive to tyrosine kinase inhibitors [23] and human prostate cancer cells sensitive to mda-7/IL-24-mediated cytotoxicity [24]. In addition, the combination of sabutoclax

and minocycline produces a synergistic cytotoxic effect in pancreatic cancer [26], and the combination of sabutoclax with a COX-2 inhibitor synergistically inhibits cell growth of oral squamous cell carcinomas [66]. Other inhibitors of pro-survival BCL-2 proteins, including ABT-737 and ABT-199, also show greater clinical efficacy in breast cancer when combined with chemotherapy or PI3K/mTOR inhibitors [56; 59]. Importantly, these combined therapies are all well tolerated. Consistent with these reports, we demonstrate that sabutoclax synergizes with cytotoxic drugs sensitizing chemoresistant breast cancer cells, suggesting that sabutoclax could be combined with standard chemotherapy for the treatment of chemoresistant breast cancer.

CSCs are critical therapeutic targets, as they generate the population responsible for therapy resistance in many cancers, including those from the breast, and their elimination may improve the outcomes of breast cancer chemotherapy [12]. Activation of the IL-6/STAT3 pathway and crosstalk between STAT3 and BCL-2 family proteins is indispensable for maintaining stemness and chemoresistance in BCSCs [47; 49]. Interestingly, the CSC population in leukemia expresses high levels of Bcl-2, and ABT-737 selectively targets CSCs in acute myelogenous and chronic myelogenous leukemia, thus establishing a strong rationale for a similar approach in solid tumors [23]. Consequently, ABT-737 inhibits the growth of lung CSCs-derived xenografts and reduces CSCs content in treated tumors [67], and sabutoclax treatment results in STAT3 dephosphorylation at Tyr705 in pancreatic cancer [26]. These observations suggest the existence of a link between IL-6/STAT3 pathways and the BCL-2 family to orchestrate drug resistance in BCSCs. Indeed, the high expression of IL-6 and STAT3 phosphorylation reported here also occurs in both chemotherapy resistant ovarian cancer [68] and multiple myeloma [69]. Importantly, SCs from chemoresistant breast cancer cells remain relatively sensitive to sabutoclax-induced cytotoxicity, in contrast to the high resistance to traditional chemotherapeutic agents. Furthermore, sabutoclax remarkably eliminates CSCs in drug resistant cells by blocking the IL-6/STAT3 pathway and the addition of IL-6 partially restores STAT3 activity and increases the CSCs population. As expected, inhibition of STAT3 activity also decreases the CSC population in drug resistant cells. Although others have also confirmed the ability of BCL-2 inhibitors to target CSCs [23; 67], as well as the decrease in stemness and therapy resistance after blocking the IL-6/STAT3 pathway [33; 47], we demonstrate that sabutoclax effectively eliminates CSCs by inhibiting IL-6/STAT3 activity both in breast cancer cells and in human breast tumor samples. Together with the demonstration of sabutoclax synergistic efficacy to overcome chemoresistance, the combination of this pan-BCL-2 inhibitor with standard chemotherapy opens new avenues for more effective therapies for treatment of relapsed breast cancer.

5. Conflict of Interest

The authors declare no conflict of interest.

6. Acknowledgements

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Figure legends

Figure 1 Sabutoclax activates apoptosis in chemoresistant breast cancer cells. (A) Misregulation of apoptotic proteins in drug resistant breast cancer cells. Protein levels of Bcl-2, Mcl-1, Bcl-xl, Bax, Bim and Puma were determined by western blots in drug naive MCF-7 and Cal51 as well as in their drug resistant derivatives MCF-7/A02 and CALDOX cells, respectively. β-actin was used as a loading control. (B) Sabutoclax IC_{50} values were obtained from drug sensitivity curves of two pairs of human breast cancer cell lines and their chemoresistant derivatives. (C) Clonogenic assays. Cells were treated with doxorubicin (3 μM for MCF-7 and MCF-7/A02; 0.2 μM for Cal51 and CALDOX) or sabutoclax (5 μM for all cell lines) for 7 days, and then stained with crystal violet. Dye was solubilized and the optical density at 592 nm was measured. (D) Apoptotic assays by flow cytometry after Annexin V/PI staining. Cells were treated with etoposide (20 μM for MCF-7 and MCF-7/A02; 2 μM for Cal51 and CALDOX) or sabutoclax (5 μM for all cell lines) for 24 h. Representative plots of three independent experiments are shown. Quantitative data show the average percentage of annexin V-positive cells (both in early apoptosis, lower right quadrant, and late apoptosis, upper right quadrant) of three independent experiments (right panel). (E) Caspase-3/7 and caspase-9 activities in drug resistant MCF-7/A02 (upper histogram) and CALDOX cells (lower histogram) after sabutoclax treatment. (F) Apoptosis-related gene (Bim, Puma, Bax and Survivin) expression levels in MCF-7/A02 and CALDOX cells determined by RT-qPCR after sabutoclax treatment for 24 h. (G) Expression levels of cleaved PARP and cleaved caspase-3 in CALDOX cells, as well as expression levels of Bim, Puma, Bax and Survivin in MCF-7/A02 and CALDOX cells determined by western blotting after sabutoclax treatment for 48 h. Numerical data are presented as mean±S.D. of three independent experiments. **P*<0.05

Figure 2 Inhibitory effect of sabutoclax on SCs in chemoresistant breast cancer cells. (A) Flow cytometry plots for CD44 and CD24 expression in MCF-7 and MCF-7/A02 cells. Cells in upper left quadrant ($CD44^{high}CD24^{low}$) are associated with SCs population. Quantitative data show the average percentage of $CD44^{high}CD24^{low}$ cells (right panel). (B) Flow cytometry analysis of ALDH activity. Cells treated with or without sabutoclax for 48 h were assayed with an Aldefluor assay kit in the presence and absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Gating in the control was set up to a maximum of 1% of cells. Representative plots of at least three independent experiments are shown. Quantitative data show the average percentage of $ALDH^{high}$ cells (right panel). (C) Mammosphere formation in chemosensitive and chemoresistant breast cancer cells treated with or without sabutoclax. Sphere forming efficacy was calculated as the number of spheres formed in 10 days divided by the original number of single cells seeded and expressed as percentage (right histogram). Representative primary mammosphere pictures are shown for each cell line (left panels). Magnification 4× for upper panel and 20× for lower panel. Spheres formed after 10 days were collected and used for Annexin V/PI staining using flow cytometry. Representative plots of three independent experiments are shown. (D) Anchorage independence was determined by the formation of clones after 3 weeks in soft agar. Data represent the average number of colonies counted in randomly chosen five visual fields under the microscope (magnification ×40). (E) Dose-response curves of sabutoclax for mammosphere cells (MCs) of MCF-7/A02 cells (upper panel) and CALDOX cells (lower panel). (F) Caspase-3/7 and

caspase-9 activities of MCF-7/A02-MC (upper histogram) and CALDOX-MC (lower histogram) after sabutoclax treatment for 24 h. Numerical data are presented as mean±S.D. of three independent experiments. *P<0.05

Figure 3 Sabutoclax diminishes BCSC population through suppression of the IL-6/STAT3 signaling pathway. (A) Western blots indicating protein levels of phosphorylated STAT3 (Tyr705) and total STAT3 in MCF-7, MCF-7/A02, Cal51 and CALDOX cells. β-actin was used as a loading control. (B) IL-6 expression at mRNA (qPCR; first and third histogram) and protein levels (ELISA; second and fourth histogram) in drug sensitive (MCF-7, Cal51) and resistant (MCF-7/A02, CALDOX) breast cancer cells. (C) Sabutoclax treatment leads to dephosphorylation of STAT3 (Tyr705). Drug resistance cells were treated with sabutoclax for 24 h and phosphorylated and total levels of STAT3 determined by western blots. β-actin was used as a loading control. (D) IL-6 expression at mRNA (qPCR; first and third histogram) and protein levels (ELISA; second and fourth histogram) in MCF-7/A02 and CALDOX cells after sabutoclax treatment for 12 h. (E) Expression levels of phosphorylated STAT3 (Tyr705) and total STAT3 in MCF-7/A02 (upper panel) and CALDOX (lower panel) cells after treatment with sabutoclax (15 μM for MCF-7/A02, 10 μM for CALDOX) and/or IL-6 (100 ng/ml for both cell lines) for 24 h. (F) Change in the SC population ($CD44^{high}CD24^{low}$) from MCF-7/A02 cells after treatment with 15 μM sabutoclax and/or 100 ng/ml IL-6 for 48 h. (G) ALDH activity of drug resistant cells treated with sabutoclax (15 μM for MCF-7/A02, 10 μM for CALDOX) and/or IL-6 (100 ng/ml for both cell lines) for 48 h. Data show the average percentage of $ALDH^{high}$ cells. (H) WP1066 blocks STAT3 phosphorylation (Tyr705) in chemoresistant breast cancer cells. Cells were treated with WP1066 (20 μM for MCF-7/A02 and 10 μM for CALDOX) for 24h h and protein levels determined by western blotting. β-actin was used as a loading control. (I) Flow cytometry analysis of CD44 and CD24 for MCF-/A02 cells (left panel) and analysis of ALDH activity for MCF-7/A02 and CALDOX cells (right panel) treated with or without WP1066 for 24h. Data show average percentage of $CD44^{high}CD24^{low}$ cells and average percentage of $ALDH^{high}$ cells.

Figure 4 Sabutoclax induces apoptosis and reduces SCs population in human breast tumors. Fresh tumor samples from nine breast cancer patients were treated *ex vivo* with 30 μM sabutoclax for 48 h in Dulbecco's modified Eagle medium and then fixed in formalin, embedded in paraffin, and sectioned for IHC staining using the corresponding antibodies. (A) IHC scores for protein expression of cleaved-caspase3, Survivin, Bax, CD44, ALDH1 and phosphorylated STAT3(Tyr705) in tissues treated with or without sabutoclax. Statistical significance was determined by a two-tailed, paired Student t test (*P<0.05). (B) Representative IHC images of tumor samples from two patients after NAC treatment that were treated *ex vivo* with or without sabutoclax.

Figure 5 Sabutoclax reduces growth of xenograft tumors and its effect is enhanced by the addition of chemotherapeutic agents. (A) Apoptotic assays by flow cytometry after Annexin V/PI staining. Cells were treated with etoposide (20 μM for MCF-7/A02, 2 μM for CALDOX) and/or sabutoclax (5 μM for both cell lines) for 24 h. Representative plots of three independent experiments are shown (left panels). Quantitative data show the average percentage of annexin V-positive cells (both in early apoptosis, lower right quadrant, and late apoptosis, upper right quadrant) of three independent experiments (right panel). (B) Colony forming assay. Drug resistant cells were treated with doxorubicin (3 μM for MCF-7/A02, 0.2 μM for CALDOX) and/or

sabutoclax (5 μM for both cell lines) for 7 days, and then stained with crystal violet. Pictorial data are representative pictures of three independent replicates. Dye was solubilized and the optical density at 592 nm was measured. (C) Tumor size (upper panel) and body weight (lower panel) of nude mice bearing MCF-7/A02 xenografts treated with PBS (control), DOX, sabutoclax or sabutoclax plus DOX. Data are presented as mean±S.D. of five mice per group. (D-E) Tumors were fixed in formalin, embedded in paraffin, and sectioned for IHC and TUNEL staining. Representative images of IHC detecting cleaved-caspase3, CD44, ALDH1 and phosphorylated STAT3 (Tyr705) are shown. Apoptosis was determined by TUNEL staining. (F) Expression levels of IL-6 determined by qPCR (left panel) in MCF-7/A02-derived tumors treated with sabutoclax or PBS, and determined by ELISA in plasma (right panel) harvested at the end point. Individual tumor expression data (dots) and mean values (lines) are indicated (*P<0.05).

Table 1 Mammosphere cells of MCF-7/A02 and CALDOX sensitivity to different drugs

Drug	IC_{50} of MCF-7/A02		Mammosphere cells resistance ratio
	Total cells	Mammosphere cells	
Doxorubicin (μM)	128.3	8827	68.8
Etoposide (μM)	206.8	8747	42.3
Sabutoclax (μM)	7.68	48.9	6.38
Drug	IC_{50} of CALDOX		Mammosphere cells resistance ratio
	Total cells	Mammosphere cells	
Doxorubicin (μM)	5.73	272.7	47.6
Etoposide (μM)	26.8	487.8	18.2
Sabutoclax (μM)	9.69	34.8	3.61

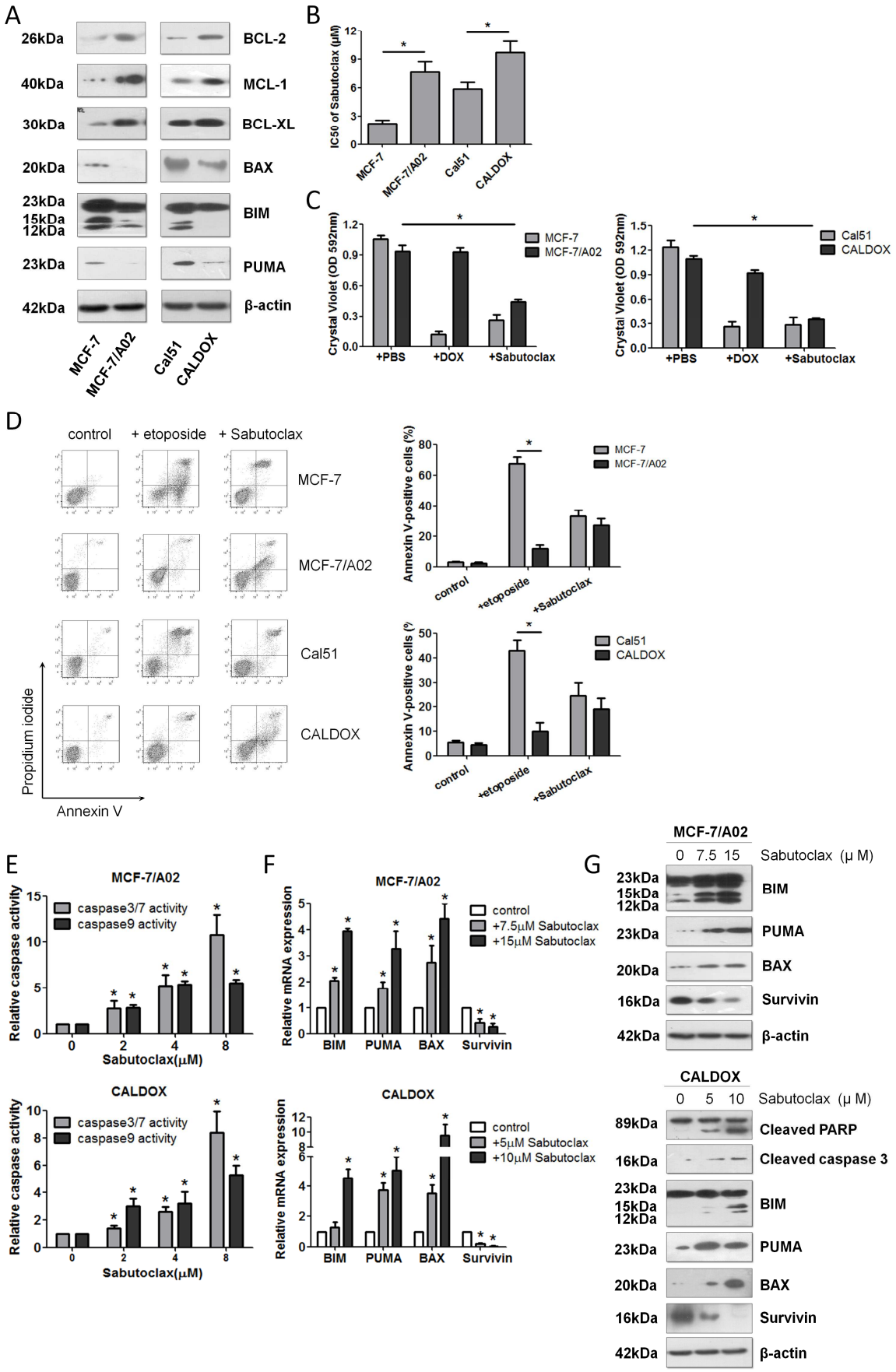
Table 2. Clinical information of the 9 patients included in the study

Characteristics	Number of patients	%
Age 51 (44-65) years		
≤ 51	5	55.6
> 51	4	44.4
Histology		
Infiltrating ductal carcinoma	6	66.7
Infiltrating (mixed) carcinoma	2	22.2
others	1	11.1
TNM stage		
I	3	33.3
II	6	66.7
Estrogen receptor status		
Negative	2	22.2
Positive	7	77.8
Progesterone receptor status		
Negative	4	44.4
Positive	5	55.6
Her2 status		
Negative	6	66.7
Positive	3	33.3

Table 3 Cytotoxicity of sabutoclax and doxorubicin/etoposide to different breast cancer cell lines

MCF-7/A02	Sabutoclax (μM)	0.781	1.563	3.125	6.25	12.5
	Doxorubicin (μM)	2.5	5	10	20	40
	CI	0.248	0.479	0.602	0.480	0.523
	Sabutoclax (μM)	0.781	1.563	3.125	6.25	12.5
	Etoposide (μM)	7.813	15.625	31.25	62.5	125
	CI	0.537	0.725	0.678	0.425	0.475
CALDOX	Sabutoclax (μM)	0.391	0.781	1.563	3.125	6.25
	Doxorubicin (μM)	0.156	0.313	0.625	1.25	2.5
	CI	0.612	0.701	0.580	0.638	0.668
	Sabutoclax (μM)	0.391	0.781	1.563	3.125	6.25
	Etoposide (μM)	0.625	1.25	2.5	5	10
	CI	0.718	0.735	0.476	0.514	0.654

Figure 1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30kDa
20kDa
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15kDa
12kDa
23kDa
42kDa
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MCL-1
BCL-XL
BAX
BIM
PUMA
β-actin
MCF-7
MCF-7/A02
Cal51
CALDOX
B
IC50 of Sabutoclax (μM)
MCF-7
MCF-7/A02
Cal51
CALDOX
C
Crystal Violet (OD 592nm)
MCF-7
MCF-7/A02
+PBS
+DOX
+Sabutoclax
Cal51
CALDOX
D
control
+ etoposide
+ Sabutoclax
MCF-7
MCF-7/A02
Cal51
CALDOX
Propidium iodide
Annexin V
Annexin V-positive cells (%)
control
+etoposide
+Sabutoclax
E
MCF-7/A02
caspase3/7 activity
caspase9 activity
Relative caspase activity
Sabutoclax(μM)
CALDOX
F
MCF-7/A02
control
+7.5μM Sabutoclax
+15μM Sabutoclax
Relative mRNA expression
BIM
PUMA
BAX
Survivin
CALDOX
control
+5μM Sabutoclax
+10μM Sabutoclax
G
MCF-7/A02
0 7.5 15 Sabutoclax (μ M)
23kDa
15kDa
12kDa
BIM
23kDa
PUMA
20kDa
BAX
16kDa
Survivin
42kDa
β-actin
CALDOX
0 5 10 Sabutoclax (μ M)
89kDa
Cleaved PARP
16kDa
Cleaved caspase 3
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15kDa
12kDa
BIM
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20kDa
BAX
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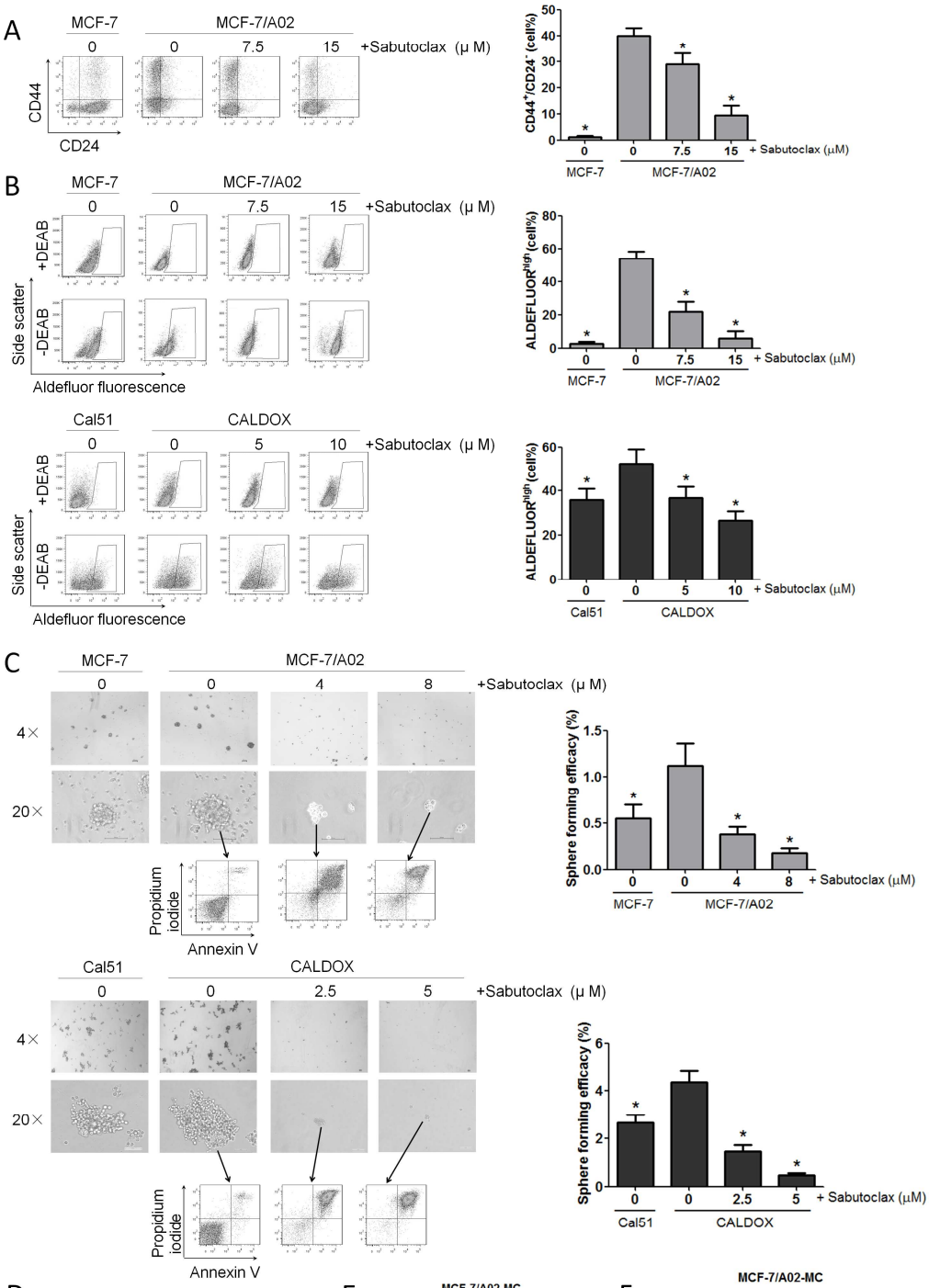
Survivin
42kDa
β-actin

Figure 2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0
7.5
15
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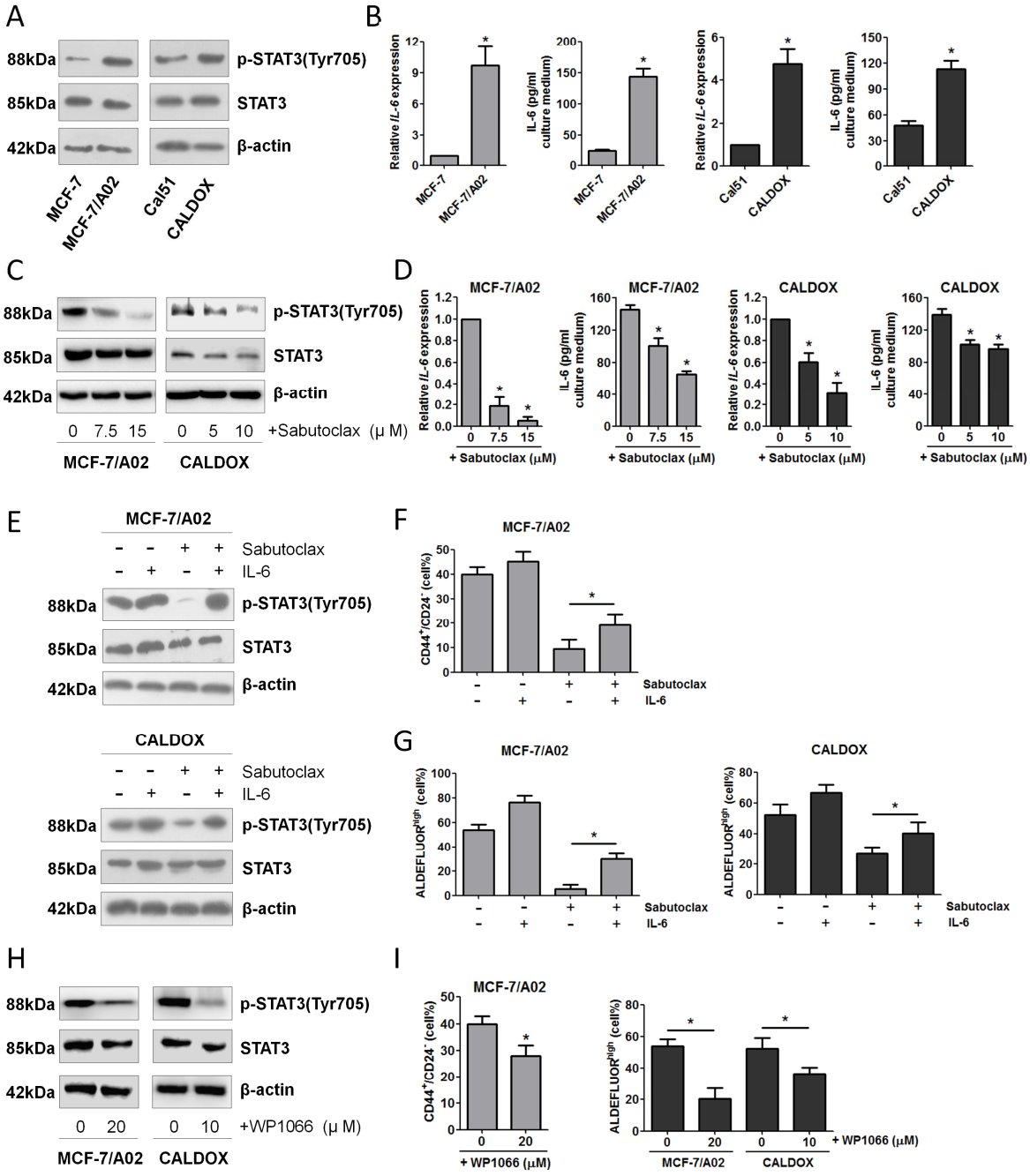
CD44
CD24
CD44+/CD24- (cell%)
+Sabutoclax (μM)
MCF-7
MCF-7/A02
B
MCF-7
MCF-7/A02
+DEAB
-DEAB
Side scatter
Aldefluor fluorescence
ALDEFLUORhigh (cell%)
Cal51
CALDOX
0
0
5
10
Cal51
CALDOX
C
MCF-7
MCF-7/A02
0
0
4
8
4×
20×
Propidium iodide
Annexin V
Sphere forming efficacy (%)
Cal51
CALDOX
0
0
2.5
5
D
Number of soft agar clones per field
E
MCF-7/A02-MC
cell survival (%)
Sabutoclax(μM)
CALDOX-MC
F
MCF-7/A02-MC
caspase3/7 activity
caspase9 activity
Relative caspase activity
Sabutoclax(μM)
CALDOX-MC

Figure 3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85kDa
42kDa
p-STAT3(Tyr705)
STAT3
β-actin
MCF-7
MCF-7/A02
Cal51
CALDOX
B
Relative IL-6 expression
IL-6 (pg/ml culture medium)
C
+Sabutoclax (μ M)
MCF-7/A02
CALDOX
D
+ Sabutoclax (μM)
E
Sabutoclax
IL-6
F
CD44+/CD24- (cell%)
G
ALDEFLUORhigh (cell%)
H
+WP1066 (μ M)
I
+ WP1066 (μM)

Figure 4

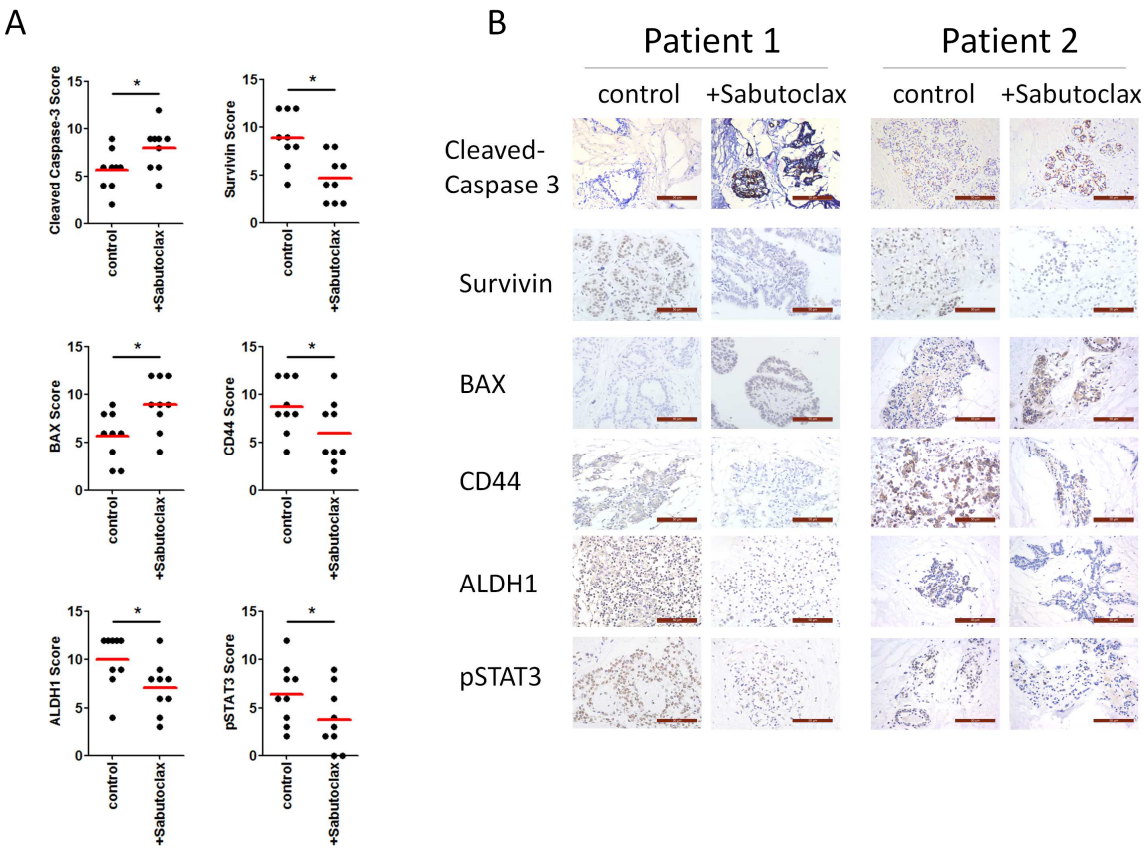
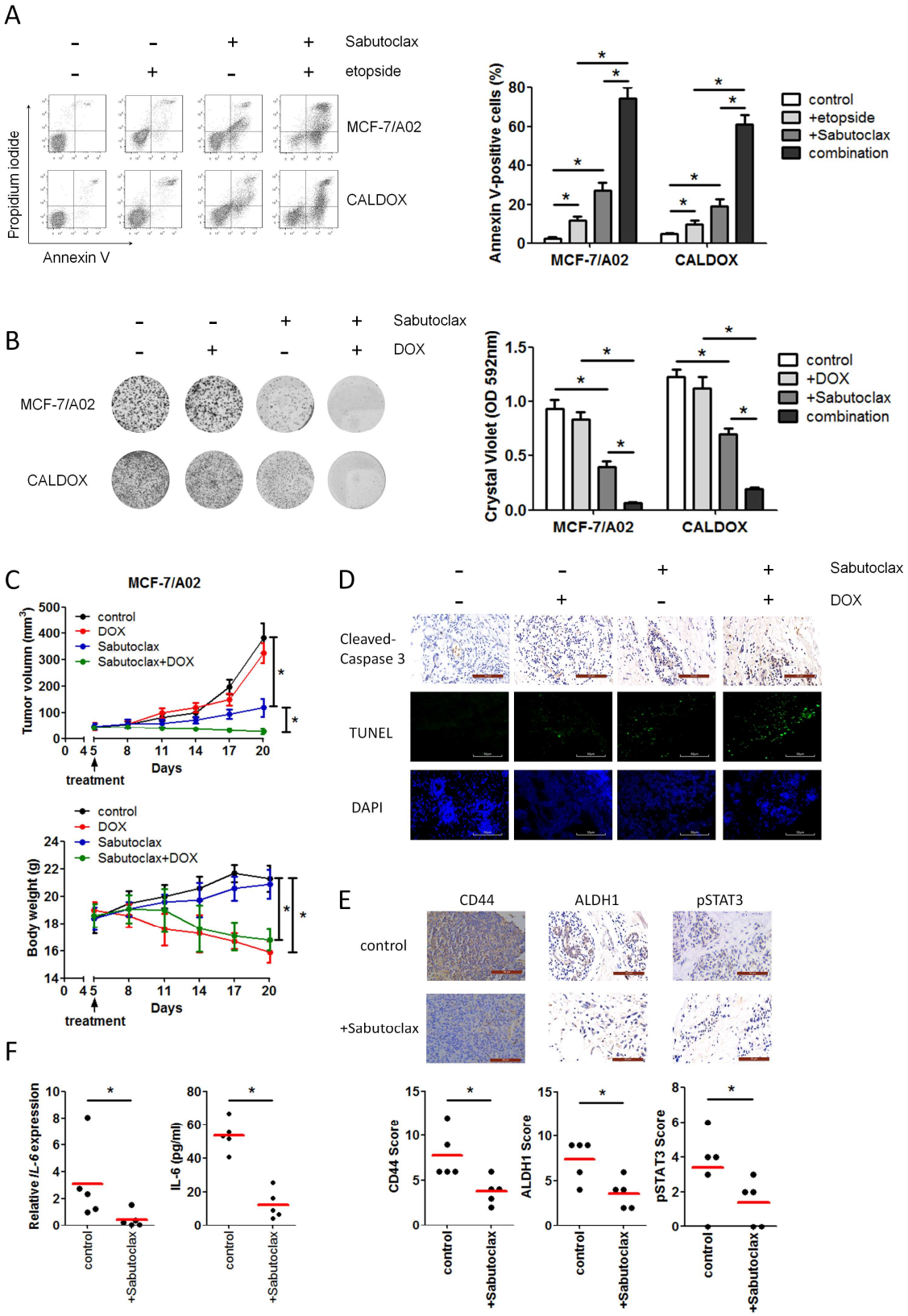


Figure 5


A
Sabutoclax
etopside
MCF-7/A02
CALDOX
Propidium iodide
Annexin V
Annexin V-positive cells (%)
control
+etopside
+Sabutoclax
combination
B
Sabutoclax
DOX
MCF-7/A02
CALDOX
Crystal Violet (OD 592nm)
control
+DOX
+Sabutoclax
combination
C
MCF-7/A02
Tumor volumn (mm3)
control
DOX
Sabutoclax
Sabutoclax+DOX
Days
treatment
Body weight (g)
D
Sabutoclax
DOX
Cleaved-Caspase 3
TUNEL
DAPI
E
CD44
ALDH1
pSTAT3
control
+Sabutoclax
F
Relative IL-6 expression
IL-6 (pg/ml)
CD44 Score
ALDH1 Score
pSTAT3 Score

- The effects of sabutoclax have been studied in chemoresistant models of breast cancer *in vitro*, *ex vivo* and *in vivo*.
- Sabutoclax was cytotoxic, led to apoptosis and elimination of cancer stem cells.
- Sabutoclax acted synergistically with chemotherapeutic agents and offers a strong rationale for its use in chemotherapy-nonresponsive breast cancer patients.