BAG3 Overexpression and Cytoprotective Autophagy Mediate Apoptosis Resistance in Chemoresistant Breast Cancer Cells

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**Abstract**

Target-specific treatment modalities are currently not available for triple-negative breast cancer (TNBC), and acquired chemotherapy resistance is a primary obstacle for the treatment of these tumors. Here we employed derivatives of BT-549 and MDA-MB-468 TNBC cell lines that were adapted to grow in the presence of either 5-Fluorouracil, Doxorubicin or Docetaxel in an aim to identify molecular pathways involved in the adaptation to drug-induced cell killing. All six drug-adapted BT-549 and MDA-MB-468 cell lines displayed cross resistance to chemotherapy and decreased apoptosis sensitivity. Expression of the anti-apoptotic co-chaperone BAG3 was notably enhanced in two thirds (4/6) of the six resistant lines simultaneously with higher expression of HSP70 in comparison to parental controls. Doxorubicin-resistant BT-549 (BT-549rDOX20) and 5-Fluorouracil-resistant MDA-MB-468 (MDA-MB-468r5-FU2000) cells were chosen for further analysis with the autophagy inhibitor Bafilomycin A1 and lentiviral depletion of ATG5, indicating that enhanced cytoprotective autophagy partially contributes to increased drug resistance and cell survival. Stable lentiviral BAG3 depletion was associated with a robust down-regulation of Mcl-1, Bcl-2 and Bcl-xL, restoration of drug-induced apoptosis and reduced cell adhesion in these cells, and these death-sensitizing effects could be mimicked with the BAG3/Hsp70 interaction inhibitor YM-1 and by KRIIB11, a selective transcriptional inhibitor of HSF-1. Furthermore, BAG3 depletion was able to revert the EMT-like transcriptional changes observed in BT-549rDOX20 and MDA-MB-468r5-FU2000 cells. In summary, genetic and pharmacological interference with BAG3 is capable to resensitize TNBC cells to treatment, underscoring its relevance for cell death resistance and as a target to overcome therapy resistance of breast cancer.

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Introduction

Despite advances in screening techniques leading to early detection of breast cancer, resistance to tumor therapy is still a major challenge in treatment of this disease, and recurrence rates are very high [1,2]. Drug resistance is broadly classified into two types: 1) de novo (intrinsic) drug resistance in patients that do not respond to conventional therapies, and 2) acquired resistance in patients developed during treatment [3]. Intrinsic and acquired therapy resistances are major challenges for the successful treatment of patients, in particular those with triple-negative breast cancer (TNBC) [4]. TNBC is a subtype of epithelial breast cancer that doesn’t express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [5]. Only 15-20% of the total population of breast cancers is triple negative, but these are highly aggressive and metastatic. Due to the absence of specific therapeutic targets, treatment strategies against this tumor subtype are severely limited. As a consequence, current treatment of these tumors is restricted to chemotherapy, frequently leading to development of therapy resistance and recurrent disease [6]. Acquired drug resistance of tumor cells can be driven by a plethora of different mechanisms, like increased drug efflux, tumor cell heterogeneity, inactivation of apoptosis, increased DNA repair, angiogenesis, altered metabolism and stress-induced genetic or epigenetic alterations after drug exposure [3,7–11]. Among these mechanisms, the adaptation of cancer cells to different cellular stress conditions (as induced by anti-cancer drugs) play a particularly important role for therapy resistance. A better understanding of the underlying resistance mechanisms is urgently required to identify new targets for treatment in an aim to improve clinical outcomes of TNBC.

Resistance to cell death caused by defects in apoptotic pathways and overexpression of anti-apoptotic proteins is a general hallmark of cancer [12–14]. Pro- and anti-apoptotic members of the Bcl-2 family are key regulators of apoptotic cell death. The Bcl-2 family proteins can be classified into three subfamilies: (i) the pro-apoptotic BH3-only proteins which have only one domain in common, the alpha helical BH3 domain; (ii) the pro-apoptotic Bax-like proteins which contain three such domains (BH1,2,3) and (iii) the anti-apoptotic Bcl-2-like proteins that contain 4 homology domains (BH4-1) and are regularly overexpressed in cancer. Bax and Bak trigger mitochondrial outer membrane permeabilization (MOMP) that is required for the release of pro-apoptotic factors from the mitochondria into the cytosol. This intrinsic apoptosis pathway is kept in check by the pro-survival Bcl-2 family members (Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bfl-1) [15–17].

The Hsp70 co-chaperone and anti-apoptotic protein BAG3 (also called Bis) is a member of the Bcl-2-associated ananthogene (BAG) protein family. This highly conserved family of co-chaperone interacts with the ATPase domain of heat shock protein 70 (HSP70) through a specific structural domain – the BAG domain [18]. BAG3 regulates several key hallmarks of cancer, including cell survival, cell adhesion, metastasis, angiogenesis and regulation of proteostasis [19,20]. A key mechanism promoting its anti-apoptotic function is represented by BAG3-dependent stabilization of the pro-survival Bcl-2 family members including Bcl-2, Bcl-xL and Mcl-1, thereby supporting the anti-apoptotic function of these proteins [21,22]. BAG3 expression has been reported to be elevated in various tumors including breast cancer, and we could previously show that estrogen receptor α (ERα) regulates a non-canonical type of autophagy that involves the function of BAG3 and provides stress resistance in ERα-expressing breast cancer cells [23]. Here we investigated the cellular mechanisms promoting enhanced chemotherapeutic resistance in TNBC cells adapted to growth in the presence of the clinically relevant chemotherapeutic agents 5-Flourouracil (5-FU), Doxorubicin (DOX) and Docetaxel (DOC). We demonstrate that increased apoptosis resistance is associated with enhanced cytoprotective autophagy, elevated expression of the oncogenic co-chaperone BAG3, BAG3-dependent stabilization of pro-survival Bcl-2 proteins and induction of EMT-like changes in gene expression. We also show that genetic and pharmacological interference with BAG3 function is capable to resensitize cells to apoptosis, underscoring the relevance of BAG3 as a target to overcome therapy resistance in TNBC.

Materials and Methods

Materials

Staurosporine (STS) was obtained from Alexis Biochemicals (San Diego, CA, USA). KIRBB11 ((N(2)-(1H-indazole-5-yl)-N(6)-methyl-3-nitropyridine-2,6-diamine)) was acquired from Calbiochem (Darmstadt, Germany). Bafilomycin A1 (Baf A1), YM-1 (2-((E)-3-Ethyl-5-(3-methylbenzo[d]thiazol-2(H)-yliide)-4-oxo-thiazolidin-2-ylidene)methyl)-1-methylpyridin-1-ium chloride), p-HEMA (Poly (2-hydroxyethyl methacrylate)), Doxorubicin hydrochloride (DOX), Docetaxel (DOC), 5-Flourouracil (5-FU), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), ABT-737 and all other chemicals or biochemistrys were purchased from Sigma-Aldrich (Munich, Germany).

Cell lines and Culture

The triple negative parental human breast cancer cell lines BT-549 and MDA-MB-468 were obtained from ATCC/LGC Promochem GmbH (Wesel, Germany). The chemoresistant cell lines were established by continuous exposure to increasing concentrations of the respective drugs to the parental cell lines as previously described [24,25] and derived from the Resistant Cancer Cell Line (RCCL) collection (www.kent.ac.uk/ncms/RCCL/RCCLabouth.html). 5-Flourouracil (5-FU) resistant BT-549 and MDA-MB-468 sublines were cultivated under continuous presence of 2000 ng/ml of 5-FU and named as BT-549′5FU2000 and MDA-MB-468′5FU2000 respectively, BT-549 and MDA-MB-468 cell lines were adapted to 40 ng/ml and 20 ng/ml of Docetaxel (DOC) and named as BT-549′DOC40 and MDA-MB-468′DOC40 respectively, whereas Doxorubicin (DOX) resistant BT-549 and MDA-MB-468 cell lines were cultivated under the presence of 20 ng/ml and 200 ng/ml of DOX and named as BT-549′DOX20 and MDA-MB-468′DOX200 respectively. BT-549 parental and chemoresistant cells were cultured in Iscove modified Dulbecco′s medium (IMDM) supplemented with 10% Fetal Calf Serum (FCS), 4 mM L-glutamine, 10% Fetal Calf Serum (FCS) and ATG5.
(SHCLNG-NM_004849; TRCN0000151474; Sigma-Aldrich) were used for transduction of both the parental and resistant cell lines of BT-549 and MDA-MB-468 breast cancer cells. Five different hairpin sequences were included to target the set. (SHC002; Sigma-Aldrich), the pLKO.1-puro Non-Mammalian shRNA control plasmid DNA was used as a negative control. Transduction was executed as previously reported [26].

Cell Viability (MTT) Assay
3,000 cells suspended in 100 µl of medium were seeded per well in 96-well-plates and cultured for 24 h before onset of treatment. After completion of the treatment period, 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from 5 mg/ml of stock solution were added into 100 µl of medium in each well, followed by 3 h of incubation in a humidified 37°C and 5% CO2 incubator. Following 3 h of incubation, the medium containing MTT reagent was discarded, the formazan crystals formed after MTT treatment were solubilized by adding 100 µl of the mixture of isopropanol/1M HCl (24:1) and gently shaken for 30 min in dark condition. Then absorbance was measured at 560 nm using microplate reader (TECAN GENios, Crailsheim, Germany).

Flow Cytometry
For quantitative estimation of cell death, flow cytometry was performed as previously described [26].

SDS-PAGE and Western Blotting
SDS-PAGE and Western blotting were performed as recently reported [27]. After blocking in 5% BSA for 1 h at room temperature, the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies directed against BAG3 (rabbit, 1:5000, Abnova, Heidelberg, Germany), LC3 (rabbit, 1:1000, Thermo Fisher Scientific, Rockford, USA), p62 (mouse, 1:1000, BD Biosciences, Heidelberg, Germany), GAPDH (mouse, 1:10,000, Calbiochem) and rest were against Bcl-2, Mcl-1, Bcl-xL, HSP70, HSF-1, Bak, Bax, ATG5, Beclin-1, pFAK (Tyr397), FAK, HSP60, E-cadherin and N-cadherin which were purchased from Cell Signaling Technology (Danvers, USA) raised in rabbit and used in the dilution of 1:1000. Following incubation, primary antibodies were detected by using respective secondary antibodies coupled with infrared dyes in green (800 CW) or red (680 RD) (IRDye goat anti-rabbit or anti-mouse from LICOR Biosciences, Bad Homburg, Germany diluted in 1:10,000 in 3% BSA for 1 h at room temperature and the signals were detected using the LI-COR Odyssey Infrared Imager (LI-COR Biosciences, Bad Homburg, Germany).

Confocal Microscopy
Cells seeded on chamber slide were fixed with paraformaldehyde (4% PFA) after completion of the respective treatments and permeabilized with 0.1% Triton X-100. For assessment of Cellular morphology, cells were stained with Texas Red-X phalloidin (ThermoFisher Scientific, Darmstadt, Germany). DAPI (Applichem, Darmstadt, Germany) was used for nuclear staining in all cases. After mounting on microscope slides, cells were finally analyzed using Nikon C1i confocal microscope.

Quantitative Real-Time PCR
Total RNA isolation and quantitative real-time PCR (qPCR) was executed as previously described [28]. The StepOnePlus™ Real-Time PCR System (Life Technologies, Darmstadt, Germany) was used for the detection of fluorescence signal above the threshold (Ct) value followed by normalization of fluorescence intensity of the samples were performed to the amplification value of the control gene TATA box binding protein (TBP). All the primers for qPCR were purchased from Applied Biosystems (Darmstadt, Germany). Following primers were used for qPCR: TaqMan® Gene Expression Assay primer for BAG3(Hs00188713_m1), CDH1 (Hs01023895_m1), CDH2 (Hs00169953_m1), SNAI1 (Hs00195591_m1), SNAI2(Hs00161904_m1), TWIST1(Hs01675818_s1), TWIST2 (Hs02379973_s1).

poly-HEMA Coating for Suspension Cultures
poly-HEMA (p-HEMA) solution was prepared by suspending 1mg of p-HEMA powder in 1 ml of 95% of ethanol followed by thorough mixing at 50°C and 750 rpm in Thermomixer comfort (Eppendorf) for overnight. Then the homogenized solution was filtered and pipetted into 24 well plates and allowed to dry inside the safety hood for two days with opened lids. Before the cells were seeded, the wells were properly washed with sterile PBS and finally the cells were allowed to remain in suspension condition throughout the experiment.

Invasion (Boyden-Chamber) Assay
Before seeding the cells into the chamber, the matrigel (Corning transwell insert with 8 μm pore, Corning, Tewksbury, MA) was rehydrated with 500 μl of FCS-free medium for 2 hours inside the humidified 37°C and 5% CO2 incubator. Then approximately 750 μl of medium containing 10% FCS was added into the wells of the 24-well cell culture insert companion plate. After discarding the rehydration medium, the transwell inserts were put into the wells of the cell cultured insert companion plate carefully by avoiding air bubble. Then approximately 20,000 cells suspended in 500 μl medium containing 2% FCS were seeded into the matrigel coated insert. After 20 hours of incubation, the cells on the top of the inserts were removed by using Q-tips and the cells attached to the bottom of the inserts were fixed with methanol for 2 minutes followed by staining for another 2 minutes with 0.1% crystal violet. The chambers were washed three four times in purified water and allowed to air dry. Then 6 pictures of each matrigel were captured with a Nikon TS100 inverted microscope equipped with a charge-coupled device (CCD) camera followed by counting of stained cells by using ImageJ software. Three matrigel chambers were used for each condition and each experiment was repeated 3 times.

Migration (Scratch) Assay
400,000 cells were seeded per well in 6 well cultured plates. After 24 hours of incubation, 10 µg/ml of Mitomycin C (Sigma-Aldrich) was added for 2 hours to prevent cell proliferation. Then a scratch was made using 200 µl pipette tip followed by washing with PBS. Pictures of the scratch at 0 hour time point were taken and the same positions were captured after 20 hours of incubation. The number of cells migrated into the scratch area were counted using ImageJ software. Each experiment was repeated for 3 times.

Subcellular Fractionation
The digitonin-based subcellular fractionation technique was employed for the separation of cytosolic and mitochondrial fractions as previously described [29].
**Global Proteomics Analysis**

For global proteomic analysis, the cells were grown until ~70% confluency. For each cell line, three independent samples were taken. Samples for LC-MS/MS analysis were prepared according to Kulak et al. with minor modifications [30]. In brief, cell lysis and protein denaturation were performed by boiling the samples in 6 M GdnHCl, 100 mM Tris pH 8.5, 10 mM TCEP and 40 mM ChlAA. Proteins were digested with Lys-C for 3 hours, followed by trypptic digestion overnight. Tryptic peptides were desalted and concentrated using STAGE-Tips (Empore C18, 3M).

Peptides were separated on an easy nLC 1200 (ThermoFisher Scientific) and a 15 cm long, 75μm ID fused-silica column, which has been packed in house with 1.9 μm C18 particles (Dr. Maisch), and kept at 45°C using an integrated column oven (Sonation). Peptides were eluted by a non-linear gradient from 4% to 48% acetonitrile over 135 minutes and directly sprayed into a QExactive HF mass-spectrometer equipped with a nanoFlex ion source (ThermoFisher Scientific). Precursor ions were analyzed with a resolution of 60,000 and the 15 most abundant ions were subjected to HCD fragmentation and resulting fragments were analyzed with a resolution of 15,000. Single charged ions and ions with unassigned charge states were not taken into account for fragmentation and dynamic exclusion was set to 20 s.

Data analysis was done with MaxQuant and essentially default settings [31]. Fragment spectra were searched against the UniproT human reference proteome (version “December 2017”), with a false discovery rate of 1% on PSM and protein level and at least one unique peptide. Fold changes were determined by LFQ quantification with the "match between runs"-option being activated [32]. Statistical significant changes between parental and resistant cell-lines were determined with Perseus using a Two-sample t-test with a significance level of 0.05.

**Statistics**

Data are represented as means ± SEM. For statistical analysis, t-test (two-tailed) was applied by using (GraphPad Software, Inc., La Jolla, CA, USA). p<0.05 was considered to be statistically significant and was denoted with asterisks or hashtags.

**Results**

**BAG3 is Overexpressed in Chemoresistant Breast Cancer Cells**

In our study, the triple negative breast cancer cell line BT-549 was adapted to growth in medium containing either 2000 ng/ml of 5-FU, 40 ng/ml of DOC or 20 ng/ml of DOX; the MDA-MB-468 cell line was similarly adapted to growth in the presence of 2000 ng/ml of 5-FU, 20 ng/ml of DOC or 200 ng/ml of DOX. The 50% inhibitory concentration (IC50) value of each drug was obtained by MTT assays. Table 1 depicts the IC50 value of each drug and the level of cross-resistance of the BT-549Par (parental control cells), BT-549r5-FU2000 (adapted to 5-Fluorouracil), BT-549rDOC20 (adapted to Docetaxel), BT-549rDOX20 (adapted to Doxorubicin), MDA-MB-468Par (parental control cells), MDA-MB-468r5-FU2000 (adapted to 5-Fluorouracil), MDA-MB-468rDOC20 (adapted to Docetaxel), and MDA-MB-468rDOX20 (adapted to Doxorubicin) cell lines to the chemotherapeutic drugs 5-FU, DOX and DOX.

| Drug     | BT-549 IC50 (ng/ml) | MDA-MB-468 IC50 (ng/ml) |
|----------|---------------------|-------------------------|
| 5-FU     | 0.63 ± 0.21         | 1.16 ± 0.14             |
| DOC      | 8.12 ± 0.84         | 9.74 ± 1.02             |
| DOX      | 9.46 ± 1.62         | 116 ± 1.69              |
| 5-FU     | 5.89 ± 0.47         | 18.97 ± 0.29            |
| DOC      | 12.82 ± 1.67        | 212.56 ± 1.20           |
| DOX      | 15.97 ± 1.26        | 180.6 ± 2.09            |
| 5-FU     | 4.24 ± 1.39         | 338.47 ± 1.40           |
| DOC      | 212.56 ± 1.20       | 384.7 ± 1.40            |
| DOX      | 180.6 ± 2.09        | 338.47 ± 1.40           |

Breast cancer cell line BT-549 was adapted to grow isolatedly with 2000 ng/ml of 5-FU, 40 ng/ml of DOC and 20 ng/ml of DOX; MDA-MB-468 cell line was grown similarly with 2000 ng/ml of 5-FU, 20 ng/ml of DOC and 200 ng/ml of DOX. Then 50% inhibitory concentration (IC50) value of each drug was determined by MTT assay. The values are means of three independent experiments performed in triplicate ± SEM. p-values<0.05 were considered to be statistically significant.
levels of HSP70 and BAG3, a HSP70 co-chaperone in all the parental and chemoresistant cells. Western blot analysis revealed a pronounced increase of BAG3 and HSP70 in most of the chemoresistant cell lines, with BT-549rDOX20 (Figure 1C) and MDA-MB-468r5-FU2000 (Figure 1D) cells showing the highest expression compared to their parental control cells, respectively.

**Figure 1.** Overexpression of BAG3, HSP70 and other anti-apoptotic proteins and down-regulation of pro-apoptotic proteins in chemoresistant breast cancer cells. (A) BT-549 and MDA-MB-468 parental and chemoresistant cell lines were treated with 3 μM of apoptotic cell death inducer staurosporine (STS) and DMSO (0.1%) as control for 6 hours followed by % of total cell death and % of Annexin-V positive & PI negative cells were estimated by flow cytometry. Data are representative of three independent experiments performed in triplicate. Columns represent means ± SEM. Statistical significance; *p<0.05, **p<0.01, ***p<0.001 and ns not significant compared to controls (DMSO). (B) Western blot analysis shows anti-apoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1) are over expressed and pro-apoptotic proteins (Bax and Bak) are down regulated in chemoresistant cell lines compared to the parental counterparts. (C) BAG3 and (D) HSP70 are highly expressed in various chemoresistant cell lines. GAPDH was used as loading control. Densitometric analysis of relative BAG3 protein expression was performed in BT-549 and MDA-MB-468 parental and chemoresistant cell lines. Columns represent means ± SEM. Statistical significance; **p<0.01, ***p<0.001 compared to parental control cells.

**Attenuation of Autophagy Increases the Sensitivity of Chemoresistant Breast Cancer Cells**

One of the potential mechanisms contributing to cell survival in chemoresistance is increased autophagy [35]. To further investigate this possibility in our cell models, we explored the basal autophagy status in all the parental and chemoresistant cells as a starting point.
Most of the chemoresistant cells exhibited slightly enhanced levels of autophagy marker proteins like LC3-II, ATG5 and Beclin-1 compared to their parental controls, with the most pronounced changes observed in BT-549rDOX20 and MDA-MB-468r5-FU2000 cells (Figure 2A) that were chosen for a subsequent, more detailed analysis. Western blot analysis revealed a shift from LC3-I to LC3-II and accumulation of p62 after combined treatment with Bafilomycin A1 and the respective drugs, i.e. DOX in BT-549Par and BT-549rDOX20 cells (Figure 2B), and 5-FU in MDA-MB-468Par and MDA-MB-468r5-FU2000 cells (Figure 2C). The LC3 shift and accumulation of p62 appeared to be more prevalent in the chemoresistant cells, suggesting that the autophagic flux may be increased in these cells compared to parental controls. The effect of autophagy is highly context-dependent and it can promote either cell survival or cell death [36]. In order to evaluate the death-modulating role of autophagy in our chemoresistant cells, a combined treatment of Bafilomycin A1 and the chemotherapeutic drugs was performed. BT-549 cells were treated with 10 ng/ml of DOX (approximate IC50 of BT-549Par) and 80 ng/ml of DOX (approximate IC50 of BT-549rDOX20), MDA-MB-468 cells were treated with 1 μg/ml of 5-FU (approximate IC50 of MDA-MB-468Par) and 18 μg/ml of 5-FU (approximate IC50 of MDA-MB-468r5-FU2000) (see Table 1). Total cell death was assessed by FACS analysis. Columns represent means of three independent experiments performed in triplicate ± SEM. Statistical significance: * p<0.05, ** p<0.01, *** p<0.001 and ns not significant compared to respective controls (Ctrls); # p<0.05, ## p<0.01 and ns not significant with Baf A1 treatment compared to without Baf A1 treatment.

**Figure 2.** Induction of prosurvival autophagy in chemoresistant cells and pharmacological inhibition of autophagy augments the sensitivity of chemoresistant breast cancer cells (A) Basal level of autophagy was increased in chemoresistant cells as LC3-II, ATG5 and Beclin-1 proteins expression were increased in chemoresistant cells compared to their respective parental cells in western blot analysis. (B) Autophagic flux was determined by the accumulation of LC3-II and p62 after combined treatment of DOX (10 ng/ml) for 72 h and autophagic flux inhibitor Baf A1 (25 nM) for 4 h in both the BT-549Par and BT-549rDOX20 cells and (C) similarly in MDA-MB-468Par and MDA-MB-468r5-FU2000 cell lines after combined treatment of 5-FU (1 μg/ml) for 72 h and Baf A1 (25 nM) for 4 h. GAPDH was used as loading control. (D) Breast cancer cell lines BT-549Par and BT-549rDOX20 were treated with 10 ng/ml and 80 ng/ml of DOX for 72 h with or without Baf A1 (25 nM for 4 h), (E) similarly MDA-MB-468Par and MDA-MB-468r5-FU2000 cell lines were treated with 1 μg/ml and 18 μg/ml of 5-FU for 72 h with or without Baf A1 (25 nM for 4 h). Total cell death was quantified by Annexin V/PI double staining followed by flow cytometry. Columns represent means of three independent experiments performed in triplicate ± SEM. Statistical significance: * p<0.05, ** p<0.01, *** p<0.001 and ns not significant compared to respective controls (Ctrls); # p<0.05, ## p<0.01 and ns not significant with Baf A1 treatment compared to without Baf A1 treatment.
DOX cells (Figure 3A), as well as in MDA-MB-468Par and MDA-MB-468’s-FU2000 cells (Figure 3B). Knockdown of ATG5 decreased the LC3-II level in BT-549DOX20 (Figure 3A) and MDA-MB-468’s-FU2000 (Figure 3B) cells. Further, we found a decrease in the expression of Mcl-1 and Bcl-2 proteins following knockdown of ATG5 in BT-549DOX20 cells (Figure 3C) and also in MDA-MB-468’s-FU2000 (Figure 3D) cells. For assessment of cell death after ATG5 KD, we performed a dose escalation experiment with DOX for 72 h in the BT-549Par/sh Ctrl, BT-549Par/ATG5 KD, BT-549DOX20/sh Ctrl and BT-549DOX20/ATG5 KD cells followed by FACS analysis of total cell death. FACS data revealed a minor, but significant increase of total cell death in the BT-549DOX20/ATG5 KD cells compared to BT-549DOX20/sh Ctrl, whereas the amount of cell death in BT-549par/ATG5 KD compared to BT-549par/sh Ctrl remained unaltered (Figure 3E). These observations confirm our previous results obtained with pharmacological inhibition of autophagy by Bafilomycin A1. Collectively, we conclude that increased autophagy in chemoresistant cells acts in a cytoprotective manner and may partially contribute to enhanced therapy resistance.
**Lentiviral Depletion of BAG3 Resensitizes Chemoresistant Breast Cancer Cells to Therapy**

So far we had observed that the higher expression of BAG3 protein is associated with simultaneously increased basal levels of autophagy and enhanced apoptosis resistance in BT-549’DOX20 and MDA-MB-468’5-FU2000 cells compared to their parental counterparts. BAG3 has key functions in regulation of both autophagy and apoptosis. It maintains cellular homeostasis by selective degradation of damaged proteins after recruiting the macroautophagic pathway [38]. Importantly, it also antagonizes activation of the intrinsic apoptosis pathway through stabilization of Bcl-2 proteins [22]. To further delineate the role of BAG3 as a resistance factor in chemoresistant cells, we established stable lentiviral BAG3 knockdowns in the BT-549Par and BT-549’DOX20 cells (Figure 4A), and in MDA-MB-468Par and MDA-MB-468’5-FU2000 cells (Figure 4B), confirmed by western blot and qPCR in BT-549Par and BT-549’DOX20 (Figure 4C), as well as MDA-MB-468Par and MDA-MB-468’5-FU2000 (Figure 4D) cells. The level of LC3-II was significantly decreased in the BT-549’DOX20/BAG3 KD cells (Figure 4A) and a similar result was also obtained in MDA-MB-468-5-FU2000/BAG3 KD cells (Figure 4B). Our western blot data indicates that HSP70 and the anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-xl are decreased in BT-549’DOX20/BAG3 KD cells (Figure 4E) and MDA-MB-468-5-FU2000/BAG3 KD cells (Figure 4F) to levels comparable to those observed in parental control cells. These data suggest that interference with BAG3 is able to effectively counteract the chemoresistance-associated increase of Mcl-1, Bcl-2 and Bcl-xl expression. It was previously proposed that BAG3 may also sequester pro-apoptotic BAX in the cytoplasm and serve to inhibit its mitochondrial translocation in glioblastoma cells [21]. Consistent with this hypothesis, we found that mitochondrial BAX was increased in BAG3-depleted MDA-MB-468’5-FU2000 cells (Figure S2). To analyze whether knockdown of BAG3 KD also reactivates the sensitivity of the chemoresistant cells to the respective drugs, we performed cell death assays following DOX treatment in BT-549 cell lines and 5-FU treatment in MDA-MB-468 cell lines, respectively. Indeed, our FACS data reveal that both BT-549’DOX20/BAG3 KD (Figure 4G) and MDA-MB-468’5-FU2000/BAG3 KD (Figure 4H) cells are significantly resensitized to the respective drugs, which was not observed in parental BAG3 KD cells compared to their controls.

**Drug Resensitization by Pharmacological Interference with the HSF1/HSP70/BAG3 Pathway**

It was recently reported that BAG3 acts as a critical molecule in HSP70-modulated cancer signaling. Thus, targeting the interaction of these two molecules may represent a promising therapeutic strategy [39]. For selective disruption of the HSP70/BAG3 complex, we used YM-1 according to Colvin et al. [39]. YM-1 treatment of BT-549’DOX20 cells dramatically reduced Mcl-1 levels in a time-dependent manner (Figure 5A). Combined treatment with YM-1 and DOX applied at different concentrations synergized in limiting cell viability in BT-549’DOX20 cells, but not in the parental BT-549 cells, as confirmed by MTT assays (Figure 5B). Furthermore, combined treatment of YM-1 and DOX significantly increased cell death in BT-549’DOX20 cells, as detected by Annexin-V staining followed by FACS analysis (Figure 5C). Please note that in this experiment, PI staining could not be performed due to the autofluorescence properties of YM-1. The stress induced transcription factor heat shock factor 1 (HSF-1) acts upstream of HSP70 and its co-chaperone BAG3. Since BAG3/HSP70 interaction stabilizes Mcl-1, inhibition of HSF-1 should lead to rapid degradation of Mcl-1 [22]. To further decipher the importance of the HSF-1/HSP70/BAG3 pathway for cell death resistance, the HSF-1 inhibitor KRIIB11 was used in comparison to the experiments previously executed with knockdown of BAG3. The combined treatment of KRIIB11 and DOX significantly increased apoptosis in BT-549’DOX20/sh Ctrl cells; with overall cell death reaching a very similar extent in comparison to BT-549’DOX20/BAG3 KD cells treated with DOX (Figure 5D). The expression of BAG3, HSP70, and Mcl-1 and LC3-II proteins gradually decreased in BT-549’DOX20 (Figure 5E) and MDA-MB-468’5-FU2000 cells (Figure 5F) in a dose-dependent manner after 48 h treatment of KRIIB11.

**BAG3 Depletion Alters Cell Matrix Adhesion in Chemoresistant Breast Cancer Cells**

It was previously reported that BAG3 significantly contributed to maintenance of the cell adhesion properties [40]. To explore the effect of BAG3 knockdown on cell adhesion properties of our drug-resistant cells, we performed F-actin staining by using Texas Red-X phalloidin followed by confocal microscopy. Confocal imaging revealed strong alterations in cell morphology in BT-549’DOX20/BAG3 KD cells (Figure 6A), while no obvious morphological changes were found in the BT-549Par/BAG3 KD cells. BT-549’DOX20/BAG3 depleted cells were more rounded and loosely attached, whereas the BAG3-proficient cells were more flattened, bigger in size and tightly attached. Similar results were also obtained in MDA-MB-468-5-FU2000/BAG3 KD cells (Figure 6B). Western blot analysis revealed that the increase in FAK phosphorylation at Tyr397 residue in BT-549’DOX20 cells (Figure 6C) and MDA-MB-468-5-FU2000 cells (Figure 6D) was reduced after BAG3 knockdown. To further assess the sensitivity of the BT-549’DOX20/BAG3 depleted cells to DOX after matrix detachment, cell adhesion was hindered by coating the cell culture plates with p-HEMA, so that all cells were cultured in suspension. Cell death was enhanced in suspension cultures of BAG3-depleted BT-549’DOX20 cells after 72 h DOX treatment whereas no significant changes of cell death were noticed in the BAG3 proficient BT-549’DOX20 cells in comparison to controls (Figure 6E).

**Depletion of BAG3 Reverses EMT-Associated Expression Changes in Chemoresistant Breast Cancer Cells**

Recently the role of BAG3 in controlling epithelial-mesenchymal transition (EMT) has come to attention of the scientific community [19]. To further investigate this phenomenon in chemoresistant cells, we performed qPCR to analyze the relative mRNA expression levels of several key genes involved in EMT. qPCR data revealed that CDH1 (encoding E-cadherin) mRNA expression was strongly increased whereas CDH2 (encoding N-cadherin) and SNAI1 (encoding Snail) expression was significantly decreased in BAG3-depleted BT-549’DOX20 cells (Figure 7A) and MDA-MB-468-5-FU2000 cells (Figure 7B), consistent with the hypothesis that BAG3 promotes EMT. We also found reduced mRNA expression of genes encoding the transcription factors SNAI2 (Snail2), TWIST1 and TWIST2 in BAG3-depleted BT-549’DOX20 (Figure 5A) and similarly in MDA-MB-468-5-FU2000 cells (Figure 5B). We did not find any significant changes in the mRNA expression of the above genes in the parental BAG3-depleted cells compared to their controls, except the increase of TWIST1 mRNA expression in BAG3-depleted parental MDA-MB-468 cells (Figure 5B). To further validate our qPCR data, we performed western blot analysis revealing a strong up-regulation of E-cadherin and down-regulation of N-cadherin in
Figure 4. Depletion of BAG3 sensitizes the chemoresistant breast cancer cells by attenuating protective autophagy (A) Stable lentiviral knockdowns of BAG3 were established in both the BT-549-Par and BT-549’DOX2000 cells and (B) also in the MDA-MB-468Par and MDA-MB-468’5-FU2000 cells. Stable lentiviral transduction of empty vector was used as control (Ctrl). Knockdowns were confirmed by western blot. Knockdown of BAG3 reduced LC3-II protein expression in BT-549’DOX2000 and MDA-MB-468’5-FU2000 cells. GAPDH served as loading control in western blot. (C) Relative BAG3 mRNA expression was also determined by quantitative PCR (qPCR) after stable lentiviral knockdowns of BAG3 in BT-549Par and BT-549’DOX2000 cell lines and (D) similarly in MDA-MB-468 cells respectively. qPCR data represent means of three independent experiments ± SEM (n = 3). Significant BAG3 mRNA expression compared to parental sh Ctrls are marked by asterisks: * p<0.05, ** p<0.01 and ns not significant. Significant differences between BAG3 KD and respective sh Ctrls are denoted by hashtags: # p<0.05, ## p<0.01 and ns not significant. (E) Knockdown of BAG3 reduced the protein expression of HSP70, Mcl-1 and other anti-apoptotic proteins whereas augments the expression of pro-apoptotic proteins like Bak and Bax in BT-549’DOX2000 and (F) MDA-MB-468’5-FU2000 cells respectively. GAPDH served as loading control. (G) BT-549’DOX2000/BAG3 KD and (H) MDA-MB-468-5-FU2000/BAG3 KD cells exhibited significantly higher levels of total cell death compared to their parental counterparts after 72 hours of DOX and 5-FU treatment in a dose dependent manner respectively. 0.1% DMSO for 72 h was used as control. Cell death was determined by Annexin V/PI double staining followed by flow cytometry. Data represent means of three independent experiments ± SEM (n = 3). *p<0.05 and ns not significant of BAG3 KD compared to respective sh Ctrls.

BAG3-depleted MDA-MB-468’5-FU2000 cells (Figure 7C) which corroborated our qPCR data. To investigate whether depletion of BAG3 has any impact on cell invasion and migration properties of the chemoresistant cells, we performed Boyden-chamber assays and scratch assays. BAG3-depleted BT-549’DOX2000 cells exhibited significantly reduced invasiveness compared to the BAG3-proficient
BT-549rDOX \textsuperscript{20} cells after 20 h of incubation (Figure 7D), and similar results were also obtained in MDA-MB-468r5-FU\textsuperscript{2000}/BAG3 KD cells (Figure 7E). Our scratch assays also revealed a significantly reduced motility of BAG3-depleted BT-549rDOX\textsuperscript{20} cells (Figure S4A) and MDA-MB-468r5-FU\textsuperscript{2000} cells (Figure S4B) after 20 h incubation compared to their controls.

Figure 5. HSP70/BAG3 interaction inhibitor YM-1 and HSF-1 inhibitor KRIIB11 sensitize the chemoresistant breast cancer cells (A) Dissociation of the HSP70/BAG3 complex by YM-1 (5 \textmu M) decreased Mcl-1 expression in BT-549rDOX\textsuperscript{20} cells after 4 h, 18 h, 24 h, 48 h treatment in western blot analysis. 0.1% DMSO for 48 h was used as Control (Ctrl) for the solvent, whereas Sorafenib (Sora) 5 \textmu M for 48 h was used as positive control. GAPDH was used as loading control in western blot. (B) % of cell viability was analyzed by MTT assay in BT-549Par and BT-549rDOX\textsuperscript{20} cells after 2 h pre-treatment of 5 \textmu M of YM-1 for 48 h followed by DOX for 72 h. DMSO 0.1 % for 48 h was used as control (Ctrl) for the solvent. (C) Breast cancer cell lines BT-549Par and BT-549rDOX\textsuperscript{20} were treated with 10 ng/ml and 80 ng/ml of DOX for 72 h with or without YM-1 (5 \textmu M, 48 h), 0.1 % DMSO for 48 h was used as control (Ctrl) for the solvent. Then cell death was analyzed by Annexin-V positive staining in flow cytometry. Columns represent means of three independent experiments ± SEM (n = 3). Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001 and ns not significant compared to ctrls (0.1 % DMSO); # p < 0.05, ## p < 0.01 and ns not significant with combined treatment of YM-1 and DOX compared to DOX treatment alone. (D) Combined treatment of KRIIB11 (20 \textmu M, 48 h) and DOX significantly augment the total cell death in BT-549rDOX\textsuperscript{20}/sh Ctrl cells compared to DOX treatment alone. 0.1 % DMSO for 48 h was used as control (Ctrl) for the solvent. Cell death was determined by Annexin V/PI double staining followed by flow cytometry. Columns represent means of three independent experiments ± SEM (n = 3). Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001 and ns not significant compared to ctrls (0.1 % DMSO); # p < 0.05, ## p < 0.01 and ns not significant with combined treatment of KRIIB11 and DOX compared to DOX treatment alone. (E) KRIIB11 treatment decreased BAG3, HSP70, Mcl-1 and LC3 II in a dose dependent manner in BT-549rDOX\textsuperscript{20} and (F) MDA-MB-468r5-FU\textsuperscript{2000} cells respectively. Cells were treated with 5 \textmu M, 10 \textmu M and 20 \textmu M of KRIIB11 for 48 h. DMSO (0.1 %, 48 h) was used as control (Ctrl). GAPDH served as loading control for western blot.
Proteomic Analysis Reveals a Gene Signature Associated with Tumor Aggressiveness

The development of drug resistance is a very complex mechanism including alterations of many different processes and proteins. Thus, we performed global proteomic analysis of BT-549rDOX20 compared to BT-549Par using label-free quantification. This analysis showed that out of the 4906 reproducibly quantified proteins groups, 271 and 307 were significantly under- and overrepresented in BT-549rDOX20 compared to BT-549Par cells (Figure 8A), respectively.

The 5 most increased and decreased proteins are also depicted in Figure 6. BAG3 depletion alters cell matrix adhesion in chemoresistant breast cancer cells (A) Knockdown of BAG3 altered cellular morphology and actin cytoskeletal distribution in BT-549rDOX20 and (B) MDA-MB-468r5-FU2000 cells compared to their respective parental counterparts in confocal imaging. For staining of F-actin, Texas Red-X phalloidin was used whereas nuclei were stained with DAPI. Scale bar 10 μm. (C) FAK phosphorylation was reduced in BT-549rDOX20/BAG3 KD and (D) MDA-MB-468r5-FU2000/BAG3 KD cells respectively. GAPDH served as loading control in the western blot. Densitometric analysis of relative pFAK protein expression was performed in control and BAG3 KD of both BT-549 and MDA-MB-468 parental and chemoresistant cell lines. Columns represent means ± SEM. Statistical significance; * p<0.05, ** p<0.01, *** p<0.001 compared to controls (E) BT-549rDOX20/BAG3 KD cells cultured in suspension exhibited more sensitivity to DOX treatment in a dose dependent manner. Cell cultured dishes were coated with pHEMA to prevent cell adhesion. Water (0.1%, 72 h) was used as control (Ctrl) for the solvent. Cell death was determined by Annexin V/PI double staining followed by flow cytometry. Columns represent means of three independent experiments performed in triplicate ± SEM. Statistical significance: * p<0.05, ** p<0.01, *** p<0.001 and ns not significant compared to ctrls (0.1 % water); # p<0.05 and ns not significant with combined treatment of DOX and pHEMA compared to DOX treatment alone.

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A, the complete list of significantly changed proteins is provided as a supplemental file (Table S1). Bioinformatics analysis using the freely available STRING-platform (string-db.org);[41] was carried to analyze Gene Ontology (GO) terms that were significantly enriched among the reduced or increased proteins (Figure 8A). In total 20 and 27 KEGG pathways were significantly overrepresented among reduced and increased protein groups respectively (Figure 8B). Reduced pathways include focal adhesion and cell adhesion molecules (CAMs). Increased pathways include citrate cycle (TCA) and oxidative phosphorylation, two metabolic pathways that are correlated with tumor aggressiveness [42], but also DNA replication and cell cycle, indicating increased proliferation. The analyses of biological processes (GO:BP; Figure 8C)
revealed that among the top 10 processes which were overrepresented among the reduced proteins, extracellular matrix organization (GO.0030198) and response to stress (GO.0006950) are the highest ranking processes. Notably, other processes whose associated proteins were significantly enriched are related to regulation of apoptosis (e.g.: regulation of extrinsic apoptotic signaling pathway (GO.2001236) and regulation of apoptotic signaling pathway (GO.2001233)) and cell adhesion (e.g.: positive regulation of cell adhesion (GO.0045785) and cell-cell adhesion mediated by integrin (GO.0033631)). On the other hand many increased processes are related to an enhanced metabolism, including cellular amino acid metabolic process (GO.0006520), cellular metabolic process (GO.00444237) and also cellular respiration (GO.00453333). Other processes are related to an increased glucose and ATP metabolism (e.g.: gluconeogenesis (GO.0006094), ATP metabolic process (GO.0046034)), replication (DNA strand elongation involved in DNA replication (GO.0006271)). Basically, each of the 10 most increased and decreased single proteins found have previously been related to tumorigenesis or tumor progression (Table 2), thus exemplifying that treatment resistance is accompanied by a more aggressive phenotype.

Discussion

Here we demonstrate that overexpression of BAG3 is associated with chemotheraphy resistance of triple negative BT-549 and MDA-MB-468 breast cancer cells that were adapted to growth in the presence of the clinically applied chemotherapeutic agents 5-FU, DOX and DOC. Furthermore, we provide evidence that the increased apoptosis resistance of these cells is associated with BAG3-dependent stabilization of the pro-survival Bcl-2 family proteins Bcl-2, Bcl-xL and Mcl-1, induction of EMT-like transcriptional changes and enhanced cytoprotective autophagy that partially contributes to increased cell survival. In line with our findings, overexpression of BAG3 has been reported in several types of human cancers, such as glioblastoma [21], lung carcinoma [43], pancreatic carcinoma [44],...
leukemia [45], and thyroid carcinoma [46], compared with very low basal levels of BAG3 in non-malignant cells [43]. Consistent with the hypothesis that BAG3 may represent a key resistance factor in breast cancer, a very recent study demonstrated that enhanced BAG3 expression correlates with poor patient survival in this tumor entity [47].

All drug-adapted BT-549 and MDA-MB-468 cell lines displayed cross resistance to chemotherapy and the apoptosis inducer STS, indicating an increase in general apoptosis resistance. BAG3 expression was visibly elevated in all three resistant MDA-MB-468 lines, indicating an increase in general apoptosis resistance. BAG3 expression correlates with poor patient survival in this tumor entity [47].

In a similar fashion, lentiviral knockdown of BAG3 significantly resensitized both resistant cell lines to drug treatment, indicating that BAG3 overexpression is involved in acquired drug resistance. Interestingly, BAG3 depletion was associated with decreased levels of both LC3-I and LC3-II, indicating that BAG3 overexpression may contribute to enhanced autophagy in the resistant setting. Of note, BAG3 was previously shown to regulate total LC3 levels at the translational level, enhanced autophagy in the resistant setting. Of note, BAG3 was previously shown to regulate total LC3 levels at the translational level, possibly leading to enhanced basal autophagy [49].

The levels of the autophagy markers LC3-II, ATG5 and Beclin-1 were also found to be elevated in BT-549rDOX20 and MDA-MB-468r5-FU2000 cells. In our chemoresistant cell models, expression of both Becl-2 and Becl-1 was elevated simultaneously, suggesting that interaction of Becl-2 and Becl-1 may have no significant effect on the regulation of autophagy in these cells. This notion is consistent with previously reported findings pointing to an indirect effect of Becl-2 on autophagy that is mediated by inhibiting Bak and Bax [48]. Since pro-survival autophagy is a key mechanism underlying drug resistance of cancer cells in many paradigms [35], we investigated the potential contribution of cytoprotective autophagy in chemotherapy resistance. To this end, we applied the pharmacological autophagy inhibitor Baf A1 and established stable lentiviral knockdowns of ATG5 to block the non-selective macroautophagy pathway. In our experiments, treatment with Baf A1 and knockdown of ATG5 significantly increased apoptotic cell death after treatment with DOX and 5-FU in BT-549rDOX20 and MDA-MB-468r5-FU2000 resistant cells respectively, but not in parental cells, indicating that enhanced autophagy may indeed partially contribute to drug resistance.

In order to overcome the proposed BAG3-driven resistance, we also pharmacologically targeted the BAG3/HSP70/Mcl-1 signaling axis. The BAG3 gene is a transcriptional target of the stress-induced transcription factor HSF1. KRIBB11 was found to selectively inhibit the transcriptional activity of HSF1 [50], which is required for expression of both BAG3 and its interactor HSP70. Indeed, we could observe significant synergistic effects on cell death in the combination therapy with KRIBB11 and DOX in the BT-549rDOX20 cell line. To further scrutinize these pro-apoptotic effects of BAG3 inhibition, we employed the specific HSP70/BAG3 small molecule inhibitor YM-1 that prevents formation and function of the HSP70-BAG3 module [39]. Inhibition of BAG3 by YM-1 significantly decreased the protein levels of Mcl-1 and was able to mimic the sensitizing effect of BAG3 depletion on apoptosis after combined treatment with DOX in BT-549rDOX20 cells. These data underscore the pivotal role of the HSP70 interaction in promoting the anti-apoptotic function of BAG3 and highlight the relevance of this complex as a therapeutic target in breast cancer.

Global proteomic analysis of BT-549rDOX20 revealed major changes in several pathways/biological processes implicated in tumorigenesis and tumor progression, including cell adhesion. Besides its anti-apoptotic function, BAG3 was also suggested to support cell adhesion and motility [40], and to mediate resistance to anoikis, a special form of apoptosis induced by matrix detachment [27]. The ability of BAG3 to regulate cell
adhesion was proposed to rely on multiple interactors (e.g. PDZGEF2 and CCN1) of BAG3 through different structural domains in this context [51]. Moreover, BAG3 was implicated in cytoskeleton organization by regulating actin folding via interaction with CCT, a cytosolic chaperonin [52]. The important role of BAG3 in cytoskeleton organization was also observed in this study, as demonstrated by gross re-arrangements of the cytoskeletal structure in BAG3-depleted cells that was visualized by actin staining followed by confocal microscopy. In particular, BAG3 depletion was accompanied by a more rounded and loosely attached cellular morphology. A role of BAG3 in controlling epithelial-mesenchymal transition (EMT) was recently proposed [19]. Indeed, we observed an EMT-like expression shift in chemotherapy-resistant cells compared to parental controls in our cell models. This shift was associated with increased expression of CDH2 (N-cadherin), SNAI1 (Snail1), SNAI2 (Snail2), TWIST1 and TWIST2 and decreased expression of CDH1 (E-cadherin). Consistent with the proposed function of BAG3 in EMT modulation, BAG3 depletion induced a back shift in the expression pattern of these genes to a more epithelial like state and also reduced the invasiveness and motility in the resistant cell models.

Our findings obtained in established cell lines and their drug-resistant derivatives represent a first step in elucidating specific mechanisms of acquired therapy resistance. These data open the avenue for follow-up investigations in patient-derived cultures and in vivo models. Collectively, we demonstrate that BAG3 plays a major role for the cell death resistance of breast cancer cells and their response to chemotherapy, as well as their aggressive growth characteristics. Based on the findings of our study, we propose that by stabilizing anti-apoptotic Bcl-2 family members and aggresive growth characteristics. Based on the findings of our study, we propose that by stabilizing anti-apoptotic Bcl-2 family members and proposing EMT-like changes, the HSFI/HSP70/BAG3 pathway may play a pivotal role for therapy resistance of breast cancer. Pharmacological intervention with BAG3 and HSP70 function is an interesting approach for combined therapies aimed at synergistically inducing apoptosis in advanced breast cancer and may aid the design of new strategies aimed at overcoming the resistance to current breast cancer therapy.

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Conflict of Interest
The authors declare no conflict of interest.

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