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Inhibition of vacuolar H+ ATPase enhances sensitivity to tamoxifen via up-regulation of CHOP in breast cancer cells

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ABSTRACT

Resistance of estrogen receptor-positive breast cancer cells to tamoxifen represents a major barrier to the successful treatment of breast cancer. In the present study, we found that vacuolar H+ ATPase (vATPase) inhibitors, bafilomycin A1 and concanamycin A, sensitize tamoxifen-induced cell death. siRNA targeting ATP6V0C, a 16-kDa hydrophobic proteolipid subunit of vATPase that plays a central role in H+ transport, markedly increased cell death induced by tamoxifen. Interestingly, bafilomycin A1 induced up-regulation of DR4/DR5 and CHOP. Knock-down of CHOP by siRNA suppressed the cell death induced by bafilomycin A1 and tamoxifen, suggesting that bafilomycin A1-mediated CHOP activation sensitizes to tamoxifen. In addition, we found that bafilomycin A1 enhances TRAIL-induced cell death in breast cancer cells. Furthermore, we showed that combination of vATPase inhibitors with tamoxifen also effectively induced cell death in HER2- and ERα-overexpressing breast cancer cells. Overall, our results demonstrate that inhibition of vATPase can potentiate the apoptotic effects of tamoxifen through up-regulation of CHOP.

1. Introduction

Breast cancer is one of the most frequent cancers and is the leading cause of death in women worldwide [1,2]. Hormone therapy is an adjuvant therapy for women whose breast cancer expresses hormone receptors [3,4]. Estrogen is known to promote the growth of many breast cancers through its binding to estrogen receptor. The selective estrogen receptor antagonist tamoxifen has improved survival in breast cancer patients. Despite improvements in treatment, resistance to tamoxifen is still a major obstacle [5]. A combination therapy of tamoxifen with other drugs that cause synergistic anti-tumor effects may therefore be an attractive option in breast cancer therapy.

Vacuolar H+ ATPase (vATPase), a multi-subunit enzyme, is an ATP-driven proton pump that translocates protons from the cytoplasm into intracellular compartments and across the plasma membrane [6,7]. vATPase consists of an ATP-hydrolyzing cytoplasmic V1 complex and a proton-translocating, membrane-bound V0 complex. By regulating cellular pH balance, vATPase is implicated in various cellular functions including endocytosis and protease activation [8,9]. Cancer cells are forced to exist in a hypoxic and acidic environment, leading to increased glycolysis [10,11]. The expression of vATPase is considered to be a well-designed compensatory mechanism that allows cancer cells to survive and proliferate [12]. Therefore, vATPase inhibitors could be promising cancer treatments. Bafilomycin A1 and concanamycin A, selective vATPase inhibitors targeting subunit c in V0 (ATP6V0C), have been suggested as potential anticancer agents [13,14].

In the present study, we demonstrated that inhibition of vATPase activity by pharmacologic drugs enhanced the sensitivity to tamoxifen by up-regulating CHOP pathway in breast cancer cells. The combinatory effects of vATPase inhibitor(s) and tamoxifen were also verified in ERα/HER2-overexpressing breast cancer cells.

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Our findings suggest that inhibiting vATPase may provide a novel approach for the treatment of tamoxifen-insensitive breast cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

MCF7 and BT474 breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HER2-overexpressing MCF7 breast cancer cells were a generous gift from Dr. Incheol Shin (HanYang University, Seoul, Republic of Korea). MCF7 and HER2-overexpressing MCF7 human breast cancer cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA), and BT474 cells were maintained in RPMI1640 (Invitrogen). Tamoxifen, bafilomycin A1, and concanamycin A were purchased from Sigma–Aldrich (St Louis, MO, USA) and recombinant human TRAIL/Apo2 ligand was from Alexis Biochemicals (Coger, Paris, France). Before tamoxifen treatment, cell media was refreshed to media containing charcoal-stripped serum.

2.2. Measurement of cell viability

Cell viability was assessed using the MTT colorimetric assay as described previously [15].

2.3. Evaluation of cell death

Cells were stained with annexin V-FITC and propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) as described previously [15].

2.4. siRNA transfections

ATP6V0C siRNAs were designed by Lim et al. [16] and were synthesized from Bioneer (Daejeon, Republic of Korea). Two different regions within the ATP6V0C gene were used: ATPV60C siRNA #1, 5′-GCCTATGGCACAGCCAAGAGCGGTA-3′ and ATPV60C siRNA #2, 5′-GCCAACTCCCTGAATGACGACATCA-3′. CHOP siRNAs (sc-35437) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection experiments were performed with Lipofectamine™ 2000, according to the manufacturer's instructions (Invitrogen).

2.5. Reverse transcription-PCR analysis

Total RNA was isolated from cells using the Trizol reagent, according to the manufacturer's instructions (Invitrogen). cDNA primed with oligo dT was prepared from 2 μg of total RNA using M-MLV reverse transcriptase (Invitrogen). The following specific primers were used for PCR: ATP6V0C (5′-AGCAGATCATGAAGTCCATC-3′

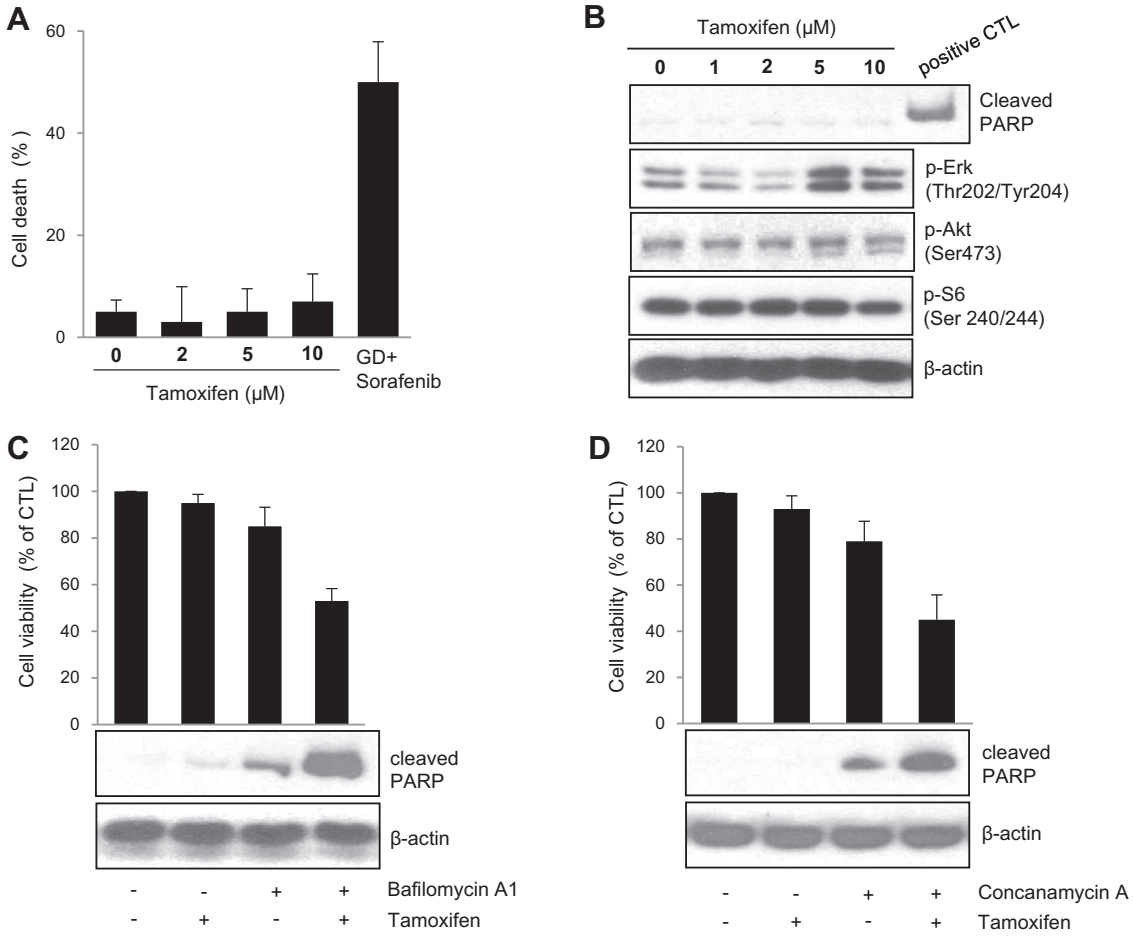

Fig. 1. V-ATPase inhibitors enhances sensitivity to tamoxifen in MCF7 cells (A, B) MCF7 cells were treated with the indicated concentration of tamoxifen for 24 h. Cell death was evaluated via flow cytometry after Annexin V and PI staining (a). Cell death data are presented as the means of triplicate samples, and error bars reflect the S.D. As a positive control, MCF7 cells were treated with 5 μM sorafenib and deprived of glucose for 24 h. (C, D) MCF7 cells were treated with either 5 nM bafilomycin A1 or 3 nM concanamycin in combination with 5 μM tamoxifen for 24 h. Cell viability was measured with the MTT assay. The viability of the control cells was set at 100%, and survival was calculated relative to this value. The indicated protein levels were estimated using western blot analysis (b–d). The blot is representative of two independent experiments. CTL; control, GD; glucose deprivation.

and 5′- GACGATGAGACCGTAGAGG-3′, 296 bp) [16], CHOP (5′-CAGACTGATCCAACTGCA G-3′ and 5′-GACTGGAATCTGGA GAGTG-3′) [17], DR4 (5′-CTGAGCAACGCAGACTCG CTGTCCAC-3′ and 5′-TCCAAGG ACACGGCAGAGCCTGTGCCAT-3′, 506 bp) [17], DR5 (5′-CGCTGCA CCAGGTGTGATTC-3′ and 5′-GTCTCCTCCACAG CTGGGAC-3′, 248 bp) [17], and β-actin (5′-GGATTCCTATGTGGGCGACGA-3′ and 5′-CGCTC GGTGAGGATCTTCATG-3′, 438 bp) [17].

2.6. Apoptosis array

The expression profiles of apoptosis-related proteins were analyzed using a human apoptosis array kit (ARY009), according with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.7. Western blot analysis

Cell lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes, followed by immunoblotting with specified primary and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Pico chemiluminescent substrates (Thermo Scientific Pierce, Rockford, IL, USA).

p-Akt at Ser473 (9271), p-Erk at Thr202/Tyr204 (9101), p-S6 at Ser240/244 (4838), and cleaved PARP (9541) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA), CHOP (ab11419) antibody from Abcam (Cambridge, UK), and the β-actin (A5316) antibody from Sigma.

3. Results

3.1. vATPase inhibitors enhanced the cytotoxic effect of tamoxifen in MCF7 cells

We first investigated the effect of tamoxifen on MCF7 cell viability. MCF7 breast cancer cells were incubated for 24 h with various concentrations of tamoxifen, and cell viability was determined by MTT assay. Cell viability was reduced to less than 20% (data not shown), despite the presence of a high concentration (10 μM) of tamoxifen [18]. Although cell death in MCF7 cells undergoing combined glucose deprivation and sorafenib treatment has been reported [15], no cell death occurred in MCF7 cells treated with tamoxifen, when assessed by annexin V/PI positivity (Fig. 1A).

Tamoxifen had no effect on Akt and S6 phosphorylation in MCF7 cells (Fig. 2B). Consistent with a previous study [19], tamoxifen markedly enhanced Erk phosphorylation. However, inhibition of Erk by PD98059, a selective inhibitor of Erk, did not induce cell sensitivity to tamoxifen (data not shown), suggesting that Erk activation is not sufficient to cause the development of tamoxifen resistance.

Bafilomycin A1 and concanamycin A are vATPase inhibitors that have been reported as potential anticancer agents [13,14]. When combining vATPase inhibitors (bafilomycin A1 or concanamycin A) with tamoxifen in MCF7 cells, we observed that cell viability was substantially reduced (Fig. 1C and D). Cell death was markedly induced in MCF7 cells by up to 45% upon co-treatment with bafilomycin A1 and tamoxifen (data not shown). PARP cleavage was also detected in cells treated with either bafilomycin A1 or concanamycin A in combination with tamoxifen (Fig. 1C and D). Our results demonstrate that combining the vATPase inhibitors with tamoxifen induces cell death to a more significant extent than does tamoxifen alone.

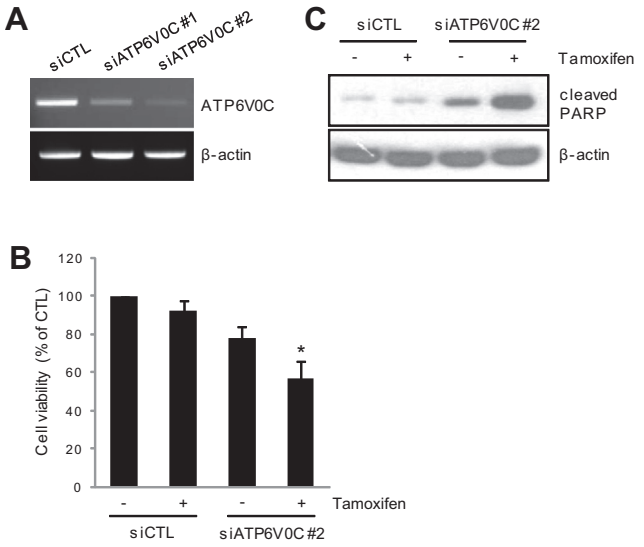

Fig. 2. Inhibition of ATP6V0C enhances sensitivity to tamoxifen in MCF7 cells (A) MCF7 cells were transfected with control or ATP6V0C siRNAs for 36 h. (B–C) MCF7 cells were transfected with control or ATP6V0C siRNA (#2) for 12 h and then treated with 5 μM tamoxifen for 30 h. The indicated mRNA (a) and protein (c) levels were estimated using RT-PCR and western blot analyses. Cell viability was measured by MTT assay (b). The viability of the control cells was set at 100%, and survival was calculated relative to this value. Cell viability data are presented as the means of triplicate samples, and the error bars reflect the S.D. $^{*}P < 0.05$ *vs* the CTL siRNA/tamoxifen-treated group. CTL; control.

3.2. Suppression of vATPase activity enhanced cell sensitivity to tamoxifen

To further establish the effect of vATPase activity on tamoxifen resistance of MCF7 cells, we transfected cells with siRNA targeting the c subunit of the V0 domain of vATPase (ATP6V0C), followed by treatment with tamoxifen. ATP6V0C siRNAs induced considerable knock-down of ATP6V0C expression in MCF7 cells (Fig. 2A) and enhanced the effects of tamoxifen in reducing cell viability and inducing PARP cleavage (Fig. 2B and C). These results clearly indicate that suppression of vATPase activity effectively enhances sensitivity to tamoxifen in MCF7 breast cancer cells.

3.3. Knock-down of CHOP induced by bafilomycin A1 suppressed the cell death induced by combination of bafilomycin A1 and tamoxifen

To clarify the underlying molecular mechanism for the enhancement of tamoxifen-induced cell death by vATPase inhibitors, the expression of apoptosis related proteins was analyzed using a human apoptosis array kit (ARY009, R&D Systems). Interestingly, the inductions of DR4 (TRAIL-R1/TNFRSF10A) and DR5 (TRAIL-R2/TNFRSF10B) proteins were observed in cells treated with tamoxifen/bafilomycin A1 (Fig. 3A). The mRNA expression levels of DR4 and DR5 were also increased by these agents (Fig. 3B).

CHOP has been identified as an upstream regulator of DR5 (17). So, we speculated that CHOP might be responsible for the DR5 up-regulation in response to bafilomycin A1 and tamoxifen treatment. As expected, expression levels of CHOP at both mRNA and protein were increased in response to bafilomycin A1 alone and combination with tamoxifen (Fig. 3C), suggesting that bafilomycin A1 mediated CHOP activation is involved in pronounced cytotoxicity to tamoxifen. To verify the role of CHOP by bafilomycin A1, we silenced CHOP expression with siRNA in MCF7 cells. As shown in Fig. 4D, knock-down of CHOP significantly suppressed the cell cytotoxicity induced by bafilomycin A1 and tamoxifen. We further investigated whether up-regulation of DR4 and DR5 by tamoxifen/

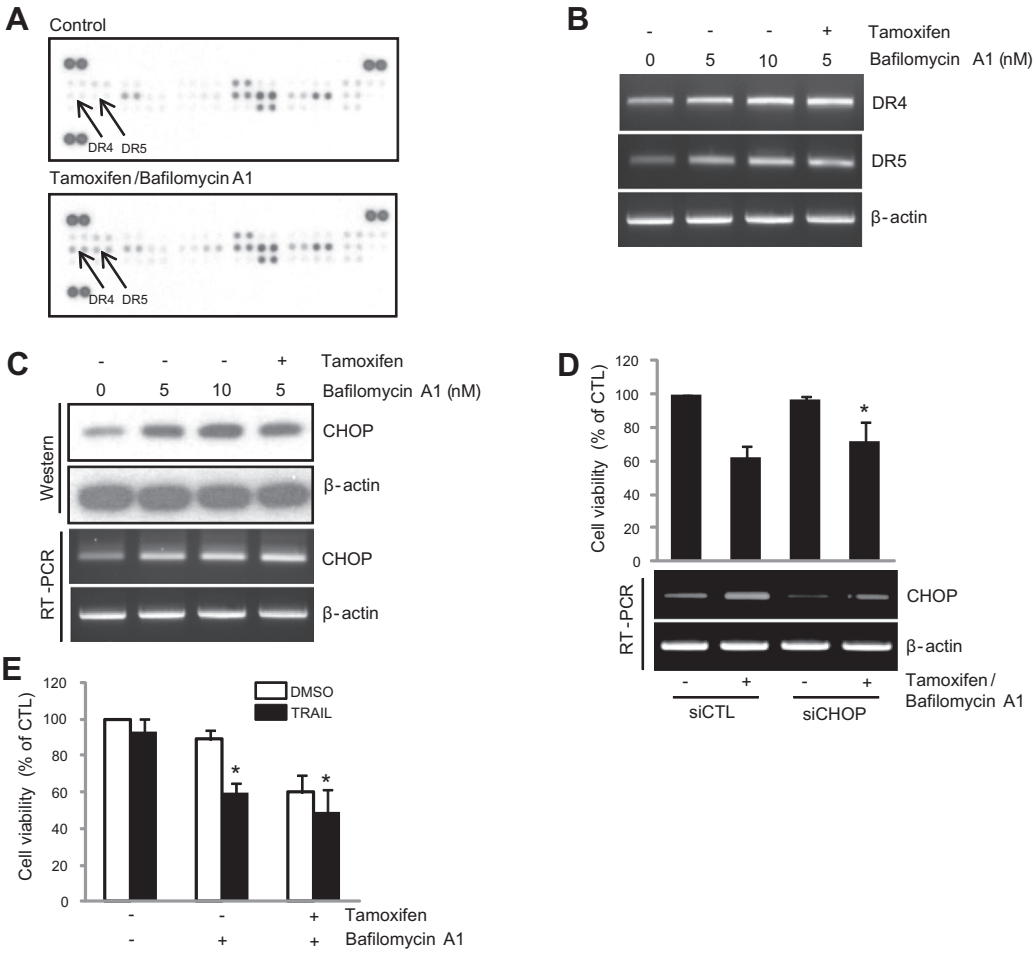

Fig. 3. Combined treatment with tamoxifen and bafilomycin A1 induces CHOP up-regulation and leads to cell death (A) Cell lysates from MCF7 cells treated with 5 μM tamoxifen and 5 nM bafilomycin A1 for 24 h were analyzed using human apoptosis array kit. (B, C) MCF7 cells were treated with the indicated concentration of bafilomycin A1 and 5 nM bafilomycin and 5 μM tamoxifen for 24 h. (D) MCF7 cells were transfected with control or CHOP siRNA for 12 h and then treated with 5 μM tamoxifen and 5 nM bafilomycin for 24 h. (E) MCF7 cells were treated with 80 ng/ml TRAIL or/and 5 nM bafilomycin or/and 5 μM tamoxifen for 24 h. The indicated mRNA (b–d) and protein (c) levels were estimated using RT-PCR and western blot analyses. Cell viability was measured by MTT assay (d, e). The viability of the control cells was set at 100%, and survival was calculated relative to this value. Cell viability data are presented as the means of triplicate samples, and the error bars reflect the S.D. $^{*}P < 0.05$ *vs* the CTL siRNA/tamoxifen/bafilomycin A1-treated group. $^{*}P < 0.05$ *vs* the bafilomycin-treated group or the tamoxifen/bafilomycin-treated group, respectively. CTL; control.

bafilomycin A1 sensitizes to TRAIL. As shown in 3E, bafilomycin A1 alone and combined treatment with tamoxifen sensitized MCF7 cells to TRAIL, whereas TRAIL alone did not induce cell death. These results strongly suggest that bafilomycin A1 could sensitize the TRAIL-induced cell death by CHOP-derived DR4 and DR5 up-regulation in MCF7 breast cancer cells.

3.4. vATPase inhibitors enhanced cell sensitivity to tamoxifen in HER2/ERα-overexpressing breast cancer cells

Overexpression and/or activation of HER family members and ERα has been associated with tamoxifen-insensitivity/resistance of breast cancer cells [20]. As shown in Fig. 4A, cell viability after tamoxifen treatment showed a slight reduction in HER2- and ERα-overexpressing MCF7 and BT474 breast cancer cells, respectively, suggesting that these cells were tamoxifen resistant. Akt and/or S6 phosphorylation levels were not changed by tamoxifen treatment (Fig. 4B). The combination of tamoxifen with vATPase inhibitors significantly reduced the cell viability of the MCF7-HER cells and BT474 cells (Fig. 4C). Taken together, combination therapy of tamoxifen with vATPase inhibitors induced synergistic cell death, even in the ER+/HER2-overexpressing breast cancer cells.

4. Discussion

Tamoxifen has become the most widely used drug in managing breast cancer [5,21]. However, as with many cancer treatments, resistance to tamoxifen is a significant issue, and up to 40% of early stage breast cancer patients who receive tamoxifen as an adjuvant therapy eventually relapse with tamoxifen-resistant disease [21].

General mechanisms have been proposed to explain the development of resistance, including continued estrogen receptor signaling in the presence of ER antagonists or the absence of estrogen [22] and the use of non-ER pathways that circumvent reliance upon ER signaling [23]. The activity of signal-transducing kinases has been implicated in both of these mechanisms [18,24], and considerable effort has been made to characterize the role of individual genes in endocrine therapy resistance, with notable findings that Pak1 and Akt activation can cause resistance to tamoxifen [25,26,27]. In our study, tamoxifen treatment in MCF7 cells led to an increase of Erk phosphorylation (Fig. 1C), and

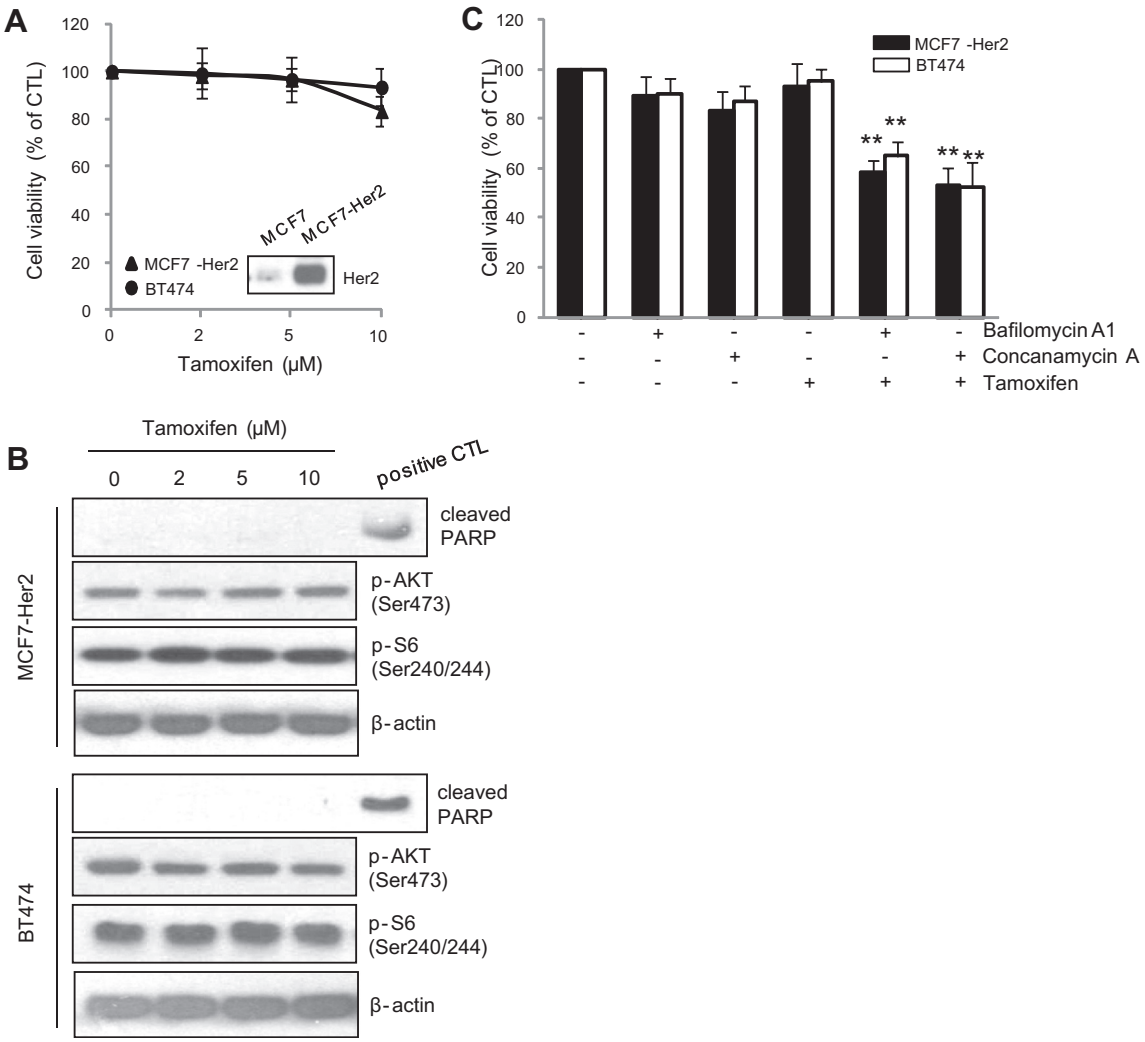

Fig. 4. V-ATPase inhibitors enhance sensitivity to tamoxifen in MCF7-HER2 and BT474 cells (A, B) MCF7-HER2 and BT474 cells were treated with the indicated concentrations of tamoxifen for 24 h. As a positive control, MCF7 cells were treated with 5 μM sorafenib and deprived of glucose for 24 h. (C) MCF7-HER2 and BT474 cells were treated with either 5 nM bafilomycin A1 or 3 nM concanamycin A in combination with 5 μM tamoxifen for 24 h. Indicated protein levels were estimated using western blot analysis (b). The blot is representative of two independent experiments. Cell viability was measured with the MTT assay (a, c). The viability of the control cells was set at 100%, and survival was calculated relative to this value. Cell viability data are presented as the means of triplicate samples, and the error bars reflect the S.D. $^{**}P < 0.01$ *vs* the tamoxifen-treated group. CTL; control.

HER2-overexpressing MCF7 cells also resulted in an increase of Erk phosphorylation (data not shown). However, inhibition of Erk signaling did not sensitize breast cancer cells to tamoxifen-induced cell death, suggesting that Erk activation is not sufficient to cause the development of tamoxifen resistance in our cell system.

vATPase is distributed widely and is found in virtually all eukaryotic cells in intracellular membranes or in the plasma membrane of specialized cells [28]. In subcellular organelles, including lysosomes, coated vesicles and endo/exocytic vesicles, vATPase is responsible for the acidification of the vesicular interior, which requires an acidic intraorganellar pH to maintain optimal enzyme activity [8,9]. Intracellular vATPases are important for receptor-mediated endocytosis and intracellular trafficking, protein processing and degradation, coupled transport of small molecules and ions, and the entry of various pathogens into cells. vATPase inhibitors bafilomycin A1 and concanamycin A have been reported as potential anticancer agents [13,14].

In the present study, the combinations of bafilomycin A1 or concanamycin A with tamoxifen further enhance cell death in MCF7 cells compared to tamoxifen treatment alone (Fig. 1). siRNA-mediated depletion of ATP6V0C increased the sensitivity of MCF7 cells to tamoxifen (Fig. 2). Interestingly, we found that bafilomycin A1 induced up-regulation of DR4/DR5 and CHOP. Knock-down of CHOP by siRNA suppressedthe cell death induced by bafilomycin A1 and tamoxifen, suggesting that bafilomycin A1-mediated CHOP activation sensitizes to tamoxifen. In addition, we found that bafilomycin A1 enhances TRAIL-induced cell death in breast cancer cells. Furthermore, combined treatment of vATPase inhibitors with tamoxifen was effective, even in ERα/HER2 overexpressing breast cancer cells. These results strongly suggest that targeting vATPase constitutes an effective chemotherapeutic approach for the treatment of tamoxifen-insensitive breast cancer cells, perhaps independently of the mechanism(s) underlying tamoxifen-resistance acquisition.

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