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Carbamazepine promotes Her-2 protein degradation in breast cancer cells by modulating HDAC6 activity and acetylation of Hsp90

Qingwei Meng · Xuesong Chen · Lichun Sun · Changhong Zhao · Guangjie Sui · Li Cai

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Abstract Histone deacetylase 6 (HDAC6) inhibition, recently, has been shown to promote the acetylation of heat-shock protein 90 (Hsp90) and disrupt its chaperone function. Her-2 oncoprotein is identified as a client protein of Hsp90. Therefore, in this study we examined the effect of carbamazepine, which could inhibit HDAC on Hsp90 acetylation and Her-2 stability. The results of this study demonstrate that while carbamazepine had no effect on the Her-2 mRNA level, it induced Her-2 protein degradation via the proteasome pathway by disrupting the chaperone function of Hsp90 in SK-BR-3 cells. Mechanistically, carbamazepine could enhance the acetylation of α-tubulin, indicating its inhibitory effect on HDAC6. Functionally, carbamazepine could synergize with trastuzumab or geldanamycin to promote Her-2 degradation and inhibit breast cancer cell proliferation. Thus, this study has potential clinical implications by providing a promising strategy to overcome the development of resistance against trastuzumab therapy for breast cancer.

Keywords Carbamazepine · Her-2 · Degradation · heat-shock protein 90 (Hsp90) · Histone deacetylase 6 (HDAC6) · Acetylation

Qingwei Meng, Xuesong Chen, and Lichun Sun have contributed equally to this study.

Q. Meng · X. Chen · L. Sun · C. Zhao · G. Sui · L. Cai (✉)
The Breast Department of the Third Affiliated Hospital of Harbin Medical University, 150040 Harbin, People's Republic of China
e-mail: caiwenxin76@yahoo.com.cn

Introduction

Overexpression of ErbB2 (Her-2) oncoprotein has been shown to promote cell transformation and is found in 25–30% of breast cancer [1, 2]. Her-2 overexpression has been positively associated with aggressive tumor phenotypes, lymph node involvement, and the resistance to chemotherapy and endocrine therapy [3, 4]. Therefore, the prognostic and therapeutic values of Her-2 have been established [5]. A recombinant humanized monoclonal antibody against Her-2, trastuzumab, was approved for the treatment of Her-2 positive breast cancer by the United States Food and Drug Administration [6]. However, about half of the patients with Her-2 overexpression do not respond to trastuzumab treatment alone or combined with chemotherapy, and nearly all patients develop the resistance to trastuzumab, leading to the disease recurrence [7]. Therefore, it is urgent to develop strategies that overcome trastuzumab resistance.

Recently, heat-shock protein 90 (Hsp90) has been recognized as an adenosine-5′-triphosphate (ATP)-dependent molecular chaperone whose client proteins include mutant p53, Bcr-Abl, Raf-1, Akt, hypoxia-inducible factor-1alpha (HIF-1alpha), Her-2, and steroid hormone receptors (reviewed in [8]). Hsp90 functions as the chaperone by mediating the formation of a multi-protein complex assisted by p50Cdc37 and p23, which binds to ATP-conjugated Hsp90 [9, 10]. Therefore, if any step of the formation of Hsp90 complex is interfered with, such as the conjugation by ATP, the chaperone function of Hsp90 will be disrupted, thus leading to the ubiquitination and degradation of the client protein. For example, Hsp90 inhibitors such as geldanamycin (GA) or its derivative, 17-allylamino-geldanamycin (17-AAG), could promote the degradation of the client protein of Hsp90, and 17-AAG is currently used in clinical trials [11].

Histone deacetylase 6 (HDAC6) is an unusual histone deacetylase, which harbors two functional catalytic domains and can be localized in the cytoplasm [12, 13]. Some recent reports have demonstrated that HDAC6 is responsible for the deacetylation of acetyl-α-tubulin and acetyl-Hsp90 [14, 15].

Carbamazepine (CBZ) is a well-known anti-epileptic drug used in clinical practice for more than four decades. Interestingly, a recent study revealed that CBZ could function as an HDAC inhibitor [16]. While many HDAC inhibitors have been confirmed as promising anti-cancer drugs, there is little information about the therapeutic effects of CBZ on cancer cells. Given the link between Hsp90, HDAC6, and Her-2, the purpose of this study is to investigate the effect of CBZ on Her-2 overexpressing breast cancer cell lines.

Materials and methods

Reagents and antibodies

CBZ, 17-AAG, trichostatin A (TSA), trapoxin B (TPX-B), MG132, sodium butyrate (SB), hydroxypropyl-beta-cyclodextrin (HBC), MTT, and ATP-Sepharose were purchased from Sigma (St. Louis, MO). The polyclonal anti-Her2 antibody was from NeoMarkers (Fremont, CA). Monoclonal antibodies to Hsp90, p21 and p27, and the polyclonal anti-HDAC6 antibody were from Santa Cruz Biotech (Santa Cruz, CA). The anti-p23 monoclonal antibody was from ALEXIS Corporation (Lausen, Switzerland). The anti-α-tubulin monoclonal antibody and anti-acetylated α-tubulin monoclonal antibody were from Sigma. The anti-acetylated lysine antibody was from Cell Signaling Technology (Beverly, MA). The anti-Hsp90 polyclonal antibody was from Stress Gen Biotechnologies Corp. (Victoria, BC, Canada). Herceptin was a gift from Sanofi-Aventis (France). The anti-β-actin monoclonal antibody was from Boster (China).

CBZ was mixed with HBC, a solubilizing agent without toxicity, at a ratio 1:10 (w:w); then, the mixture was dissolved in deionized water followed by repeated vortexing [17].

Cell culture and treatment

SK-BR-3 and MDA-MB-231 cell lines obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplied with 10% FBS at 37°C in a humidified incubator supplemented with 5% carbon dioxide. Cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and allowed to grow for 48 h before treatment as indicated.

Western blotting

Cells were harvested and lysed in ice-cold buffer (50 mMol Tris–Cl pH 7.4, 150 mMol NaCl, 1% Triton X-100, 1% Nonidet P-40, 1 mMol EDTA, 1 mMol phenylmethylsulfonyl fluoride, and a protease inhibitor mixture). Lysed cells were collected by scraping and centrifuging at 14,000 rpm for 20 min at 4°C. The supernatant was collected, and the protein concentration was determined by the Bradford method [18]. Equal amounts of protein (40 μg/lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Pall, NY). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST), and further incubated with primary antibodies for Her-2, alpha-tubulin, acetyl-α-tubulin, p21, p27, HDAC6, or beta-actin. After washing with TBST, the membrane was incubated with secondary antibodies coupled with alkaline phosphatase and developed using enhanced chemiluminescence (ECL) (Amersham, PA).

RT-PCR

Total RNA was isolated from SK-BR-3 cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using reagents supplied in an RT-PCR kit (TaKaRa, Japan) with nine random primers, and subjected to PCR amplification using primers for Her-2 and beta-actin. The primers were synthesized by TaKaRa Bio (China), and their sequences were as follows: Her-2 forward, 5′-AGCCGCGAGCACCCAAGT-3′; reverse, 5′-TTGGTGGGC AGGTAGGTGAGTT-3′; amplicion: 147 bp. Human beta-actin forward, 5′-TGACGGGGTCACCCACACTGTGC CCATCTA-3′, reverse 5′-CTAGAAGCATTTGCGGT GGACGATGGAGGG-3′; amplicion: 661 bp. Amplification conditions were 35 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C.

Immunoprecipitation

Cells were lysed in the lysis buffer (50 mMol Tris, 2 mMol EDTA, 100 nMol NaCl, 1 mMol sodium orthovanadate, 25 mMol NaF, and 1% Triton X-100, pH 7.5), and total proteins were quantified using the Bradford assay. Approximately 500 μg of total protein was incubated with 2 μg primary antibody (1:100) at 4°C for 2 h. 20 μl protein G agarose beads was added to the mixture and incubated overnight at 4°C. The immunoprecipitated complexes were washed thrice with the lysis buffer, resuspended in the SDS sample loading buffer, and subjected to Western blot analysis using an anti-Hsp90 or anti-p23 antibody. Alternatively, cell lysates were prepared after drug treatment,

and Hsp90 was affinity-precipitated from 200 μg of total protein using ATP-sepharose beads, then subjected to Western blot analysis using a monoclonal anti-Hsp90 antibody [19].

MTT assay

Cell proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were plated onto 96-well plates (2×10^3 cells/well, 200 μl cell suspension per well) and cultured overnight. Cells were then treated with CBZ (100 μMol), 17-AAG (5 μMol) [11], herceptin (20 μg/ml), CBZ + 17-AAG, or CBZ + herceptin. After incubation for 24 h, 20 μl of PBS containing MTT (0.5 mg/ml) was added to each well and incubated for another 4 h at 37°C. The supernatant was discarded and 150 μl of DMSO was added. When the stain was dissolved, the optical density absorbance value (ABS) of each well was read on a microplate reader at 490 nm.

Results

CBZ promotes the degradation of Her-2 protein

First, we examined the effect of CBZ on the Her-2 protein level in SK-BR-3 cells. As shown in Fig. 1a, the Her-2 protein significantly decreased after a 24-h treatment with 10 and 100 μMol CBZ. The Her-2 protein level began to decrease after 8 h of treatment with CBZ and continued to decrease with treatment up to 24 h (Fig. 1b). Furthermore, the CBZ-induced decrease of the Her-2 protein level was prevented by MG132, an inhibitor of proteasome (Fig. 1d). Taken together, these data have suggested that CBZ promotes the degradation of the Her-2 protein in a dose- and time-dependent manner.

Next, we performed an RT-PCR assay to determine whether CBZ could affect the transcription of Her-2. After 24-h treatment with CBZ at various concentrations, nearly no difference in Her-2 mRNA levels could be observed (Fig. 1c). Thus, these data have indicated that CBZ modulates Her-2 protein at the post-translation level.

CBZ induces acetylation of Hsp90 and inhibits its ability to bind ATP

Following the treatment of SK-BR-3 cells with CBZ for 12 h, Hsp90 was immunoprecipitated with ATP-sepharose from cell lysates, and the levels of Hsp90 were examined by Western blot analysis. CBZ, 17-AAG, and TSA [20] (but not SB [20, 22]) were effective in disrupting the ATP-binding ability of Hsp90 (Fig. 2a). To determine whether the impaired ATP-binding ability was caused by the acetylation of Hsp90, we performed another immunoprecipitation assay with CBZ-treated cells in various concentrations. Notably, we found that CBZ treatment could elevate the acetylation level of Hsp90 without changing the total Hsp90 (Fig. 2b). In another set of experiments, we compared the effects of CBZ treatment on the acetylation level of Hsp90 with the effects of TSA or SB treatment, since TSA and SB are histone deacetylase inhibitors but the latter cannot inhibit HDAC6 [20]. We found that CBZ and TSA but not SB could acetylate Hsp90 (Fig. 2c). This point was also supported by a breakdown of p23 binding to Hsp90 with treatment of CBZ, 17-AAG, or TSA, but not with SB (Fig. 2d). According to McLaughlin [21], p23 binding to ATP-bound Hsp90 is essential to a stable ATP-dependent conformational state and high affinity for client proteins.

CBZ is an inhibitor of HDAC6

On the basis of the data described above, we hypothesized that CBZ could be an inhibitor of HDAC6. Therefore, we chose a special HDAC6 substrate, α-tubulin, for the following experiments. We observed that CBZ, in contrast to SB and TPX-B [20], could enhance the acetylation of α-tubulin (Fig. 3a). Because there is evidence that changes in expression of p21 or p27 are involved in HDAC inhibitor-induced cytotoxicity [22], we investigated whether expression of p21 and p27 would be affected by treatment with CBZ or SB. Unexpectedly, almost no change could be detected in p21 and p27 after treatment with 100 μMol CBZ for 24 h (Fig. 3b). Conversely, p21 but not p27 significantly increased with SB treatment (Fig. 3c). In addition, the levels of HDAC6 were examined in SK-BR-3 cells treated with CBZ at various concentrations, and no changes were detected (Fig. 3d).

CBZ synergizes with other drugs to promote Her-2 degradation and inhibit breast cancer cell proliferation

In another experiment, Her-2 overexpressing cells were exposed to treatment with CBZ, or herceptin, or CBZ plus herceptin. We observed that Her-2 protein decreased more significantly in the cells treated with both drugs than in cells treated with either drug alone (Fig. 4a). In addition, CBZ and 17-AAG were also synergistic in promoting Her-2 protein degradation (Fig. 4b).

Next we examined the effects of combined treatment on breast cancer cell proliferation and found that while CBZ treatment alone had no effect on cell proliferation, a combination of CBZ with herceptin or 17-AAG significantly inhibited cell proliferation (Fig. 4c).

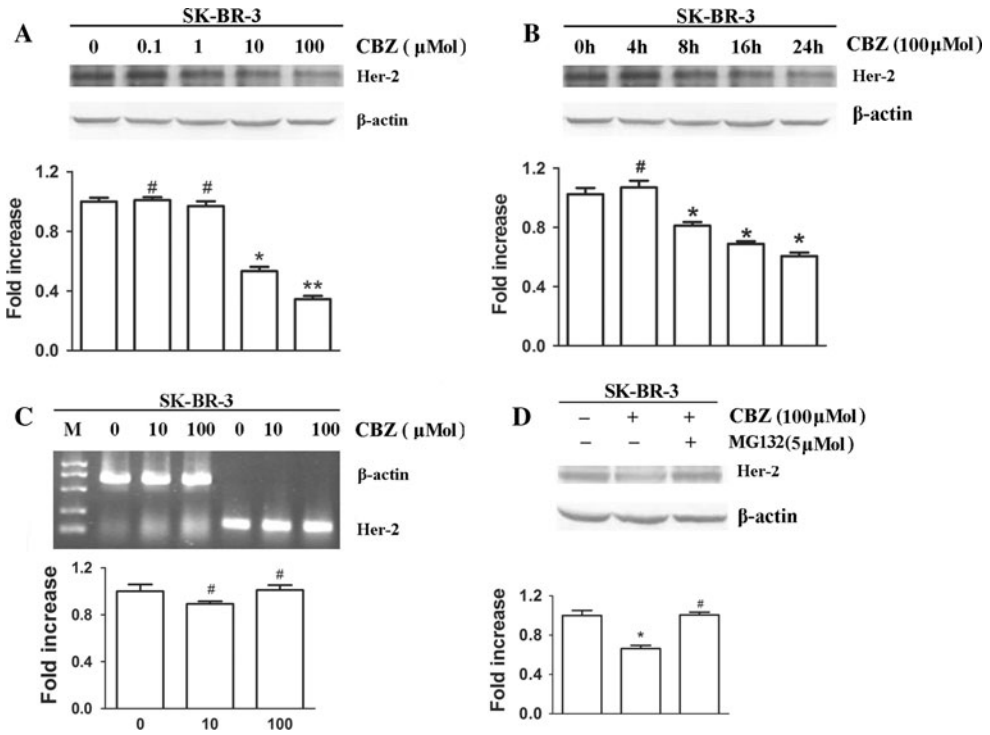

Fig. 1 CBZ promotes Her-2 protein degradation in breast cancer cells. **a**, **b** SK-BR-3 cells were treated with CBZ at the concentration indicated for 24 h (**a**) or treated with 100 μMol CBZ for various times (0, 4, 8, 16, or 24 h) (**b**). The cell lysates were immunoblotted with anti-Her2 polyclonal antibody. *β*-actin served as the loading control (**c**). SK-BR-3 cells were treated with CBZ at the concentration indicated. Total RNA was isolated and subjected to RT-PCR assay. The PCR products were resolved on a 1.8% agarose gel and stained with ethidium bromide. *M* DNA marker (**d**). SK-BR-3 cells were treated with CBZ or CBZ plus MG132. The cell lysates were immunoblotted with anti-Her2 polyclonal antibody. *β*-actin served as the loading control. $^{\#}$ $P > 0.05$, * $P < 0.05$, and ** $P < 0.01$

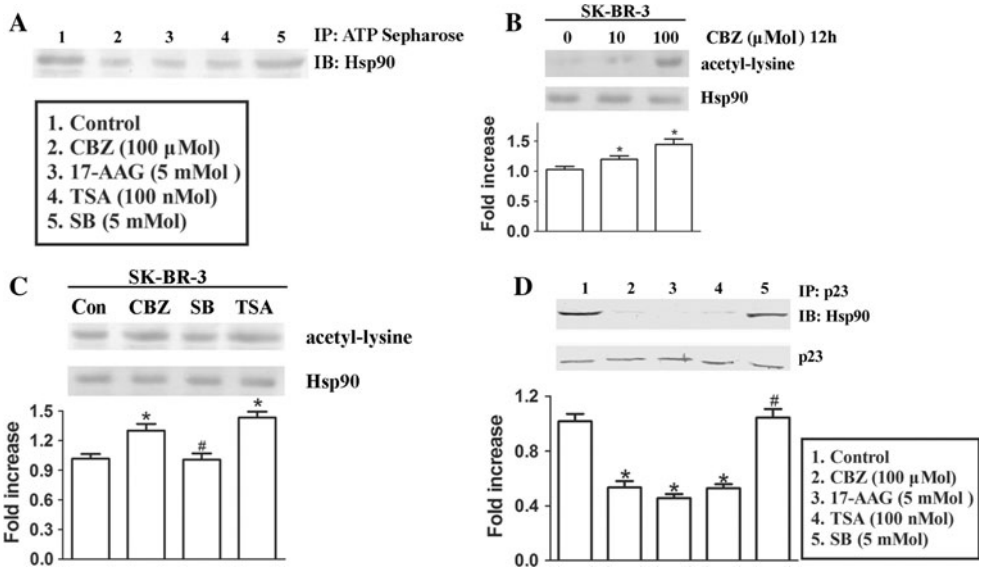

Fig. 2 CBZ disrupts the ATP-binding ability of Hsp90 and increases the acetylation of Hsp90 (**a**). SK-BR-3 cells, untreated, or treated with 100 μMol CBZ, 5 μMol 17-AAG, 100 nMol TSA and 5 mMol SB for 12 h, then Hsp90 was affinity-precipitated from cell lysate with ATP-sepharose beads. Hsp90 was detected using an anti-Hsp90 specific antibody (**b**). SK-BR-3 cells, untreated, or treated for 12 h with CBZ at the concentration indicated, then Hsp90 was immunoprecipitated from cell lysate with anti-Hsp90 antibody followed by immunoblotting with anti-Hsp90 or anti-acetylated lysine antibody (**c**). SK-BR-3 cells, untreated, or treated with 100 μMol CBZ, 5 mMol SB, 100 nMol TSA for 12 h. Hsp90 was immunoprecipitated from cell lysate with anti-Hsp90 antibody followed by immunoblotting with anti-Hsp90 or anti-acetylated lysine. $^{\#}$ $P > 0.05$, * $P < 0.05$

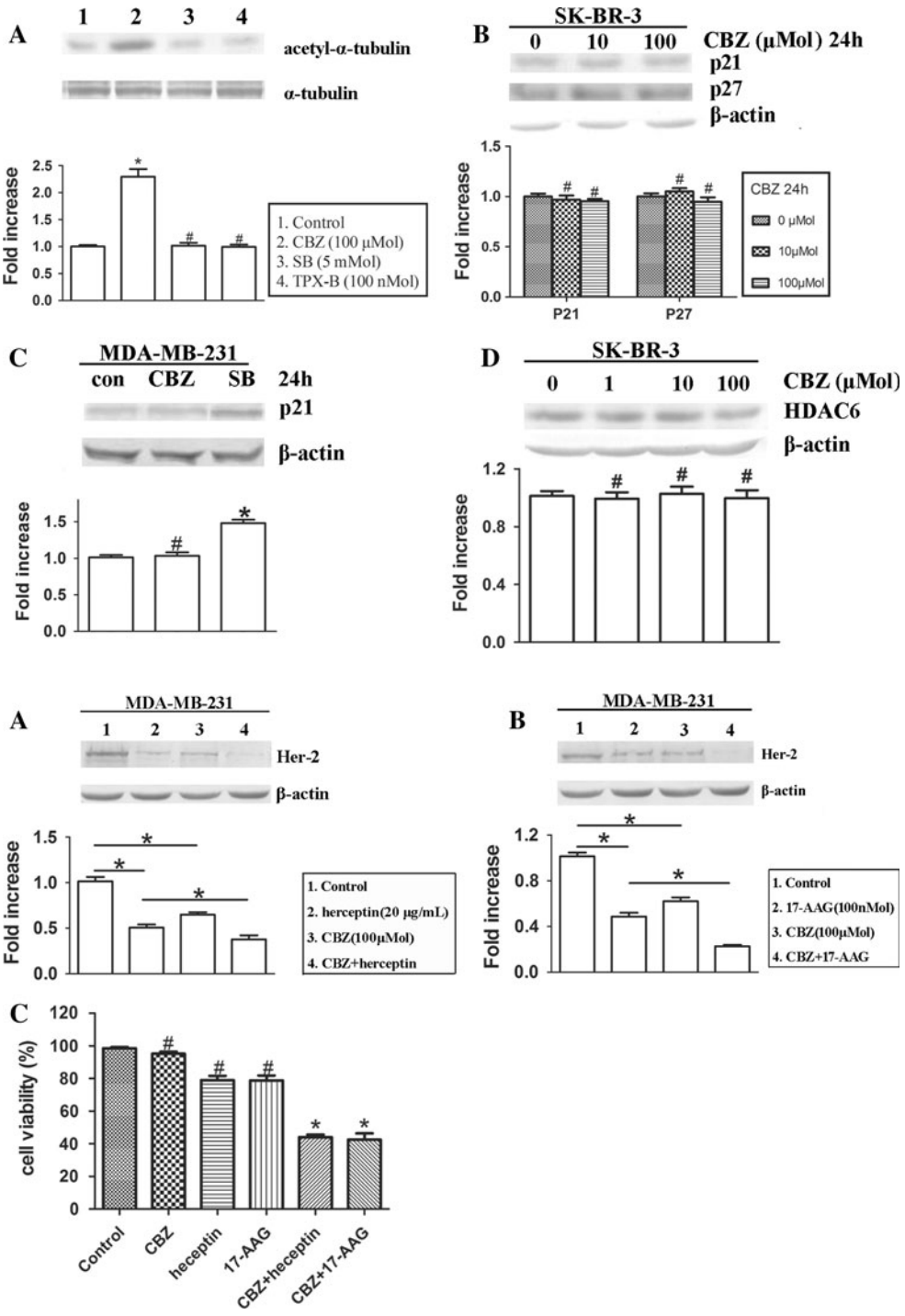

Fig. 3 CBZ enhances the acetylation of α-tubulin (**a**). SK-BR-3 cells were treated with 100 μMol CBZ, 5 mMol SB, or 100 nMol TPX-B for 24 h. Western blots were performed to detect the levels of acetyl-α-tubulin and total α-tubulin (**b**). SK-BR-3 cells were treated with CBZ at various concentrations for 24 h. Anti-p21 and anti-p27 antibodies were used to evaluate expression of p21 and p27 (**c**). MDA-MB-231 cells, untreated, or treated with 100 μMol CBZ or 5 mMol SB for 24 h. The level of p21 was detected using Western blots (**d**). SK-BR-3 cells were treated with CBZ at various concentrations. Anti-HDAC6 antibody was used to detect HDAC6 expression. β-actin served as the loading control. $^{\#}$ $P > 0.05$, * $P < 0.05$

Fig. 4 CBZ synergizes with 17-AAG or herceptin to promote Her-2 degradation and inhibit breast cancer cell proliferation (**a**, **b**). MDA-MB-231 cells were treated as indicated for 24 h. Western blots were performed using polyclonal anti-Her2 antibody to detect the Her-2 level (**c**). MDA-MB-231 cells were treated as indicated for 24 h. MTT assay was performed to evaluate cell proliferation. Means ± SD were calculated from six individual experiments. $^{\#}$ $P > 0.05$, * $P < 0.05$

Discussion

In this study, we examined the effect of CBZ on the Her-2 protein level in breast cancer cell lines. For the first time, we report here that CBZ treatment can reduce Her-2 protein in a Her-2-overexpressing SK-BR-3 breast cancer cell line. Interestingly, CBZ had no effect on the mRNA level of Her-2, and the proteasome inhibitor MG132 could reverse CBZ-induced downregulation of Her-2. Collectively, these results provide strong support that CBZ induces Her-2 degradation via the proteasome pathway.

It is noteworthy that a variety of proteins or drugs can induce ubiquitin-mediated degradation of Her-2 [23]. Especially, Her-2 has been shown as a client protein of Hsp90 and its degradation is regulated by Hsp90 [24, 25]. In line with these results, we found that CBZ could impair

Hsp90's chaperone function by hampering the access to ATP. Moreover, an augmentation of Hsp90 acetylation was also observed following CBZ treatment. These findings indicate that CBZ treatment results in the acetylation of Hsp90; as a consequence, the acetyl-Hsp90 loses the ATP conjunction site. In fact, reversible acetylation of Hsp90 has already been verified as a functionally important post-translational modification of Hsp90 [19], and treating cells with an HDAC inhibitor can cause acetylation of Hsp90 and depletion of several Hsp90 client proteins [26]. Consistent with these studies, the results of this study suggest that CBZ treatment induces increased acetylation of Hsp90 via inhibiting HDACs, thus leading to the disassociation of Her-2 and Hsp90 and the access of Her-2 to ubiquitin-mediated degradation.

So far, among all the HDAC members, only HDAC6 is identified to function as Hsp90 deacetylase [15, 27]. Although a previous study suggested that CBZ was an inhibitor for HDAC3 and HDAC7 [16], we postulate that it could also inhibit HDAC6. Hence, we investigated whether CBZ treatment caused hyperacetylation of α-tubulin, a cytosolic protein mainly acetylated by HDAC6. Indeed we found a higher level of acetyl-α-tubulin after CBZ treatment. Nevertheless, it is important to consider that HDAC6 is unlikely to be the only Hsp90 deacetylase since HDACi FK228, which does not inhibit HDAC6, was employed in the initial studies that identified Hsp90 hyperacetylation [28, 29]. But, in this study, we did not observe the expected increase in p21 following HDAC inhibition by CBZ, possibly because it is not strong enough as an inhibitor against HDAC class I proteins to affect the expression of other proteins. Apart from its HDAC- inhibitory activity, CBZ exerts its clinical effects mainly by impacting on voltage-sensing ion channels [30], and this function may be more decisive.

Furthermore, although we did not observe a significant inhibition of breast cancer cell proliferation by CBZ treatment, we found that CBZ can synergize with trastuzumab to further downregulate Her-2 protein and inhibit breast cancer cell proliferation. Thus, this study has potential clinical implications in that it provides a promising strategy to overcome the development of resistance against trastuzumab therapy.

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