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# Ubiquitination and Downregulation of ErbB2 and Estrogen Receptor-Alpha by Kinase Inhibitor MP-412 in Human Breast Cancer Cells

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## ABSTRACT

ErbB2 has been proven to be an important target for breast cancer therapy. MP-412 is a dual ErbB2 and epidermal growth factor receptor tyrosine kinase inhibitor belonging to an irreversible-type anilinoquinazoline derivative. We demonstrate herein that along with the kinase inhibition, MP-412 has the ability to induce ubiquitination, internalization, and degradation of ErbB2 in several human breast cancer cell lines at concentrations relatively higher than those required for kinase inhibition. Another irreversible inhibitor, CI-1033, showed similar activity, while the reversible compounds were ineffective, suggesting a crucial role of covalent bonding functionality in these effects. In MCF7 cells, MP-412 depleted not only ErbB2 but also estrogen receptor (ER)-α, and to some extent, affected Raf-1, while MP-412 activated Hsp70 expression. Moreover, we observed that MP-412 increased immunocomplexing of Hsp70 with ErbB2 and ER-α, with simultaneous induction of ubiquitination of these client proteins. Furthermore, in combination with proteasome inhibitor, MP-412 resulted in the noticeable accumulation of ErbB2 and ER-α in the detergent insoluble fraction of cell lysates. These results suggest that MP-412 acts as an inhibitor of Hsp90 function, whereas MP-412 did not bind directly to ATP-binding site of Hsp90, unlike geldanamycin. We also found that new protein synthesis was involved in the activity of MP-412 on Hsp90 modulation. Since downregulation of ErbB2 and ER-α by accelerating the ubiquitin-proteolysis system will become an attractive approach for breast cancer therapy, we expect MP-412 to be a lead compound for the drug design and the development of such agents. J. Cell. Biochem. 112: 2279–2286, 2011. 

**KEY WORDS:** ErbB2; ESTROGEN RECEPTOR-ALPHA; Hsp90; MP-412; UBIQUITIN

Metastatic breast cancer is the leading cause of cancer-related deaths among women worldwide [Parkin et al., 2005]. Overexpression of ErbB2 (Her2/Neu), a member of the epidermal growth factor receptor (EGFR) family, has been observed in up to 30% of breast cancer patients and is associated with a poor prognosis [Slamon et al., 1987]. Inhibition of ErbB2 signaling has emerged as a promising anticancer strategy. In fact, the humanized monoclonal antibody trastuzumab that targets ErbB2 has successfully achieved prolongation of the survival in patients with ErbB2-positive metastatic breast cancer [Slamon et al., 2001]. However, even in patients with strongly ErbB2-positive tumors, the response rate to trastuzumab remains limited. Therefore, alternative agents effective to ErbB2-expressing tumors are still desired. The oral dual kinase inhibitor GW-2016 (lapatinib), which acts on both ErbB2 and EGFR, has recently been approved by the US Food and Drug Administration for treatment of patients with ErbB2-positive metastatic breast cancer [Bulletin Board, 2010]. Moreover, heat shock protein 90 (Hsp90) inhibitors, such as 17-allylamino-demethoxygeldanamycin (17-AAG) and 17-*N*,*N*-dimethyl ethylene diamine-geldanamycin (DMAG), are currently under clinical investigation for treatment of metastatic breast cancer, because ErbB2 is one of the most sensitive client proteins to Hsp90 inhibitors [Kamal et al., 2004; Brandt and Blagg, 2009].

Hsp90 is a molecular chaperone that plays a regulatory role in refolding and trafficking in protein maturation, interfering with the activity, and stability of many client proteins such as kinases and steroid hormone receptors [Kamal et al., 2004]. Indeed, Hsp90 is reportedly required for the stability of ErbB2 and estrogen receptor

Abbreviations: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; CHIP, carboxy terminus of Hsc70-interacting protein; CHX, cycloheximide; DMAG, 17-*N*,*N*-dimethyl ethylene diamine-geldanamycin; DMSO, dimethylsulfoxide; EGFR, epidermal growth factor receptor; ER-α, estrogen receptor-α; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; Ub, ubiquitin.

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(ER)-α, and geldanamycin causes rapid degradation of them mediated by the ubiquitin-dependent proteasome system [Xu et al., 2001; Fan et al., 2005]. Estrogen-dependent breast cancer usually progresses from an antiestrogen-sensitive to an antiestrogen resistant state. Indeed, in ER-positive breast cancers, overexpression of EGFR and ErbB2 is associated with resistance to antiestrogen therapy [Kurokawa et al., 2000].

MP-412 is a dual ErbB2 and EGFR tyrosine kinase inhibitor belonging to an irreversible-type anilinoquinazoline class of compounds, and it shows significant antitumor activity in both ErbB2- and EGFR-overexpressing cancer cells in preclinical models [Suzuki et al., 2007, 2009]. We have previously reported that MP-412 inhibits cellular autophosphorylation of EGFR and ErbB2 with $IC_{50}$ values of 43 and 282 nM, respectively [Suzuki et al., 2007]. In this study, we are focusing on the distinct pharmacological characteristics of this compound apart from its kinase inhibitory activity. We herein describe that MP-412 downregulates Hsp90 client proteins including ErbB2 and ER-α through the mechanism of ubiquitin-proteolysis system in human breast cancer cells.

## MATERIALS AND METHODS

### CELL CULTURE

The human breast cancer cell lines T-47D, MCF7, and SK-BR-3 were purchased from American Type Culture Collection (Rockville, MD). The cells were cultured in DMEM-Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified 5% $CO_2$ incubator.

### ANTIBODIES AND CHEMICALS

The following antibodies were used: c-ErbB-2 (NeoMarkers, Fremont, CA), Akt, p44/42 extracellular signal-regulated kinase (Erk), phospho-Akt S473, phospho-Erk T202/Y204, and phospho-ErbB2 Y1221/1222 (Cell Signaling Technology, Beverly, MA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Trevigen, Gaithersburg, MD), Hsp70 (Stressgen Bioreagents, Victoria, BC, Canada), and ER-α, ubiquitin (Ub), and Raf-1 (Santa Cruz, Santa Cruz, CA). Anti-ER-α antibody for immunoprecipitation was purchased from NeoMarkers. MP-412, CI-1033 (canertinib), GW-2016 (lapatinib), PS-341 (bortezomib), and ZD-1839 (gefitinib) were synthesized at Mitsubishi Tanabe Pharma Corporation (Yokohama, Japan). Cycloheximide (CHX) and ammonium chloride were purchased from Sigma (Saint Louis, MO). 17-*N*,*N*-dimethyl ethylene diamine-geldanamycin (DMAG) was purchased from InvivoGen (San Diego, CA).

### IMMUNOPRECIPITATION AND WESTERN BLOTTING

Cell lysis and immunoblotting were performed as described previously [Suzuki et al., 2007]. For immunoprecipitation, cells were lysed in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% CHAPS, 1 mM $Na_3VO_4$, and protease inhibitor cocktail), and 300–500 μg of lysates were incubated with anti-ErbB2 or anti-ER-α antibody (2 μg/mg protein) at 4°C for 6 h, followed by the addition of protein G-Sepharose™ beads (GE Healthcare, Uppsala, Sweden) and rotation at 4°C overnight. The beads were washed with lysis buffer, resuspended in SDS sample buffer, boiled and then loaded onto SDS-PAGE. For preparation of detergent-soluble and detergent-insoluble fractions, cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM EDTA, 1 mM $Na_3VO_4$, and protease inhibitor cocktail), and the insoluble fraction (pellet) was solubilized by sonication twice for 10 s each in SDS buffer (2% SDS, 10 mM dithiothreitol, 5 mM EDTA, 1 mM $Na_3VO_4$, and protease inhibitor cocktail).

### FLOW CYTOMETRY

In brief, after treatment with the drugs, cells were harvested and resuspended in PBS containing 1% bovine serum albumin (BSA, Sigma) with 1 μg/ml of trastuzumab (Chugai Pharmaceutical, Tokyo, Japan) or control human IgG (MP Biomedicals, Solon, OH) and then with an R-Phycoerythrin-conjugated anti-human IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and analyzed on a Cytomics FC500 flow cytometer (Beckman-Coulter, Fullerton, CA).

### CONFOCAL IMMUNOFLUORESCENCE MICROSCOPY

The cells grown on coverslips were treated with the drugs for 6 h and with Hoechst 33342 (AnaSpec, San Jose, CA) for the last 20 min at 37°C, and then fixed and permeabilized simultaneously in Cytofix/Cytoperm™ solution (BD Biosciences, Franklin Lakes, NJ). The cells were stained with 1 μg/ml of Alexa Fluor® 488-labeled trastuzumab on ice for 30 min followed by extensive washing in PBS. The fluorescent images were obtained using Nikon C1si confocal microscopy (Nikon, Tokyo, Japan).

### Hsp90 COMPETITIVE BINDING ASSAY

Recombinant human Hsp90-α (BPS Bioscience, San Diego, CA) and fluorescence-labeled geldanamycin (BPB Bioscience) both at a final concentration of 5 nM in assay buffer (20 mM HEPES, pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 0.01% NP-40, 0.1 mg/ml bovine serum albumin, 2 mM dithiothreitol) was added in 384-well black plate. Dimethylsulfoxide control or test compounds were added and the plate was incubated for 2 h at room temperature. Then fluorescence polarization was measured using a Spectrofluorometer (Molecular Devices, Sunnyvale, CA) with an excitation of 485 nm and emission of 530 nm.

## RESULTS

### KINASE INHIBITOR MP-412 HAS AN ABILITY TO INDUCE UBIQUITINATION AND DOWNREGULATION OF ErbB2

We examined first the ability of MP-412 to inhibit phosphorylation signaling of ErbB2 in human breast cancer cell line T-47D. In western blotting, MP-412 showed complete inhibition of ErbB2 autophosphorylation as well as the consequent activation of Akt and Erk at 1 μM for only 1 h while both DMAG and PS-341 did not affect ErbB2 phosphorylation within this short period of time (Fig. 1A). Since we earlier noticed that overnight exposure of MP-412 depleted ErbB2 proteins in cells, we examined the time course of ErbB2 expression after MP-412 treatment in T-47D cells and revealed that ErbB2 expression was noticeably downregulated by 6 h with 10 μM of MP-412, concomitant accumulation of ubiquitinated proteins in

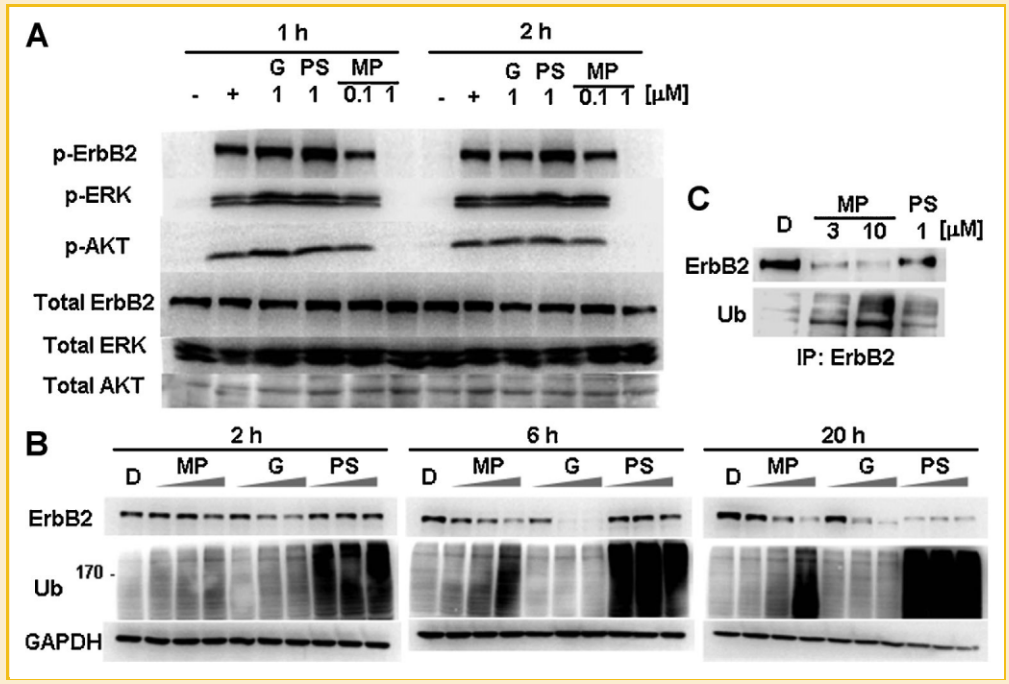


Fig. 1. MP-412 inhibits ErbB2 phosphorylation and induces ubiquitination and downregulation of ErbB2 in T-47D cells. A: Serum-starved cells were exposed to MP-412 (MP), DMAG (G), and PS-341 (PS) at the indicated concentrations for 1 or 2 h and were stimulated with 100 ng/ml heregulin-β1 for 5 min. B: Cells were treated with DMSO (D), MP-412 (MP, 1, 3, and 10 μM), DMAG (G, 0.1, 0.3, and 1 μM), or PS-341 (PS, 0.1, 0.3, and 1 μM) for 2, 6, or 20 h. Cells were lysed, and protein extracts were analyzed by western blotting with the indicated antibodies. C: Cells were treated with DMSO, MP-412, or PS-341 for 6 h before cell lysis. ErbB2 proteins were immunoprecipitated as described in the Materials and Methods section, and the immunoprecipitates were analyzed by western blotting with anti-ErbB2 and anti-ubiquitin antibodies. All experiments presented in this and other figures were repeated at least three times.

whole-cell lysates (Fig. 1B). In contrast, DMAG depleted ErbB2 completely by 6 h with prior minimal increase in protein ubiquitination, and PS-341 increased protein ubiquitination by 2 h, whereas it took overnight incubation for ErbB2 depletion (Fig. 1B). Ubiquitination of ErbB2 in whole cell lysate after MP-412 treatment was confirmed by anti-ubiquitin immunoblotting of ErbB2-immunoprecipitates (Fig. 1C). We also observed the ubiquitination and downregulation of ErbB2 by MP-412 in another cell line MCF7 (data not shown). These results indicated that MP-412 has the ability to induce ubiquitination and downregulation of ErbB2 at concentrations relatively higher than those required for kinase inhibition.

### IRREVERSIBLE, BUT NOT REVERSIBLE, KINASE INHIBITORS INDUCE UBIQUITINATION, INTERNALIZATION, AND DEGRADATION OF ErbB2

We next explored whether other existing tyrosine kinase inhibitors of anilinoquinazoline derivatives showed similar biological activities in order to clarify their chemical functionalities. Both MP-412 and CI-1033 contain an acrylamide substituent, which can covalently bind to the thiol moiety of cysteine residues in the kinase domain of ErbB2 [Fry et al., 1998; Suzuki et al., 2007]. In contrast, GW-2016 and ZD-1839 are reversible inhibitors that lack the Michael reaction (nucleophile) acceptor, and aMP is an amine-reduced analog of MP-412 whose acrylamide moiety is replaced by a primary amine, lacking the covalent bonding ability but retaining its phosphorylation inhibitory activity (data not shown). MP-412 and CI-1033 reduced ErbB2 expression at 10 μM in T-47D cells, whereas the reversible inhibitors, GW-2016, ZD-1839, and aMP, were ineffective even at 30 μM (Fig. 2A). Moreover, in the ErbB2-overexpressing cell line SK-BR-3, MP-412 induced the ubiquitination and downregulation of ErbB2 at 10 μM, and CI-1033 showed similar activity at 30 μM, whereas the reversible inhibitors showed no effect (Fig. 2B). When we performed confocal immunofluorescence microscopy with anti-ErbB2 antibody to determine how MP-412 downregulated the receptor, MP-412 remarkably reduced cell-surface staining of ErbB2, instead of the appearance of internalized immunostaining spots in the cytosol at 10 μM (Fig. 2C). Since the compound at 1 μM, which is enough concentration to inhibit tyrosine phosphorylation, showed neither internalization nor reduction of ErbB2 immunostain suggesting that these events were independent of its kinase inhibition. Although DMAG reduced strikingly ErbB2 in cell surface by 6 h, we were able to detect only a minuscule increase in intracellular aggregates of ErbB2 and its ubiquitination (Fig. 2B and C). Those might be due to the fact that ErbB2 polyubiquitination occurred within minutes of exposure to geldanamycin directed to the rapid degradation of the receptor [Mimnaugh et al., 1996]. Only CI-1033 but not GW-2016, ZD-1839, or PS-341 showed less abundant internalization of ErbB2 than MP-412 (Fig. 2C). Collectively, when comparing between reversible and irreversible inhibitors, it is suggested that the covalent bonding functionality of MP-412 plays a crucial role in the ubiquitination,

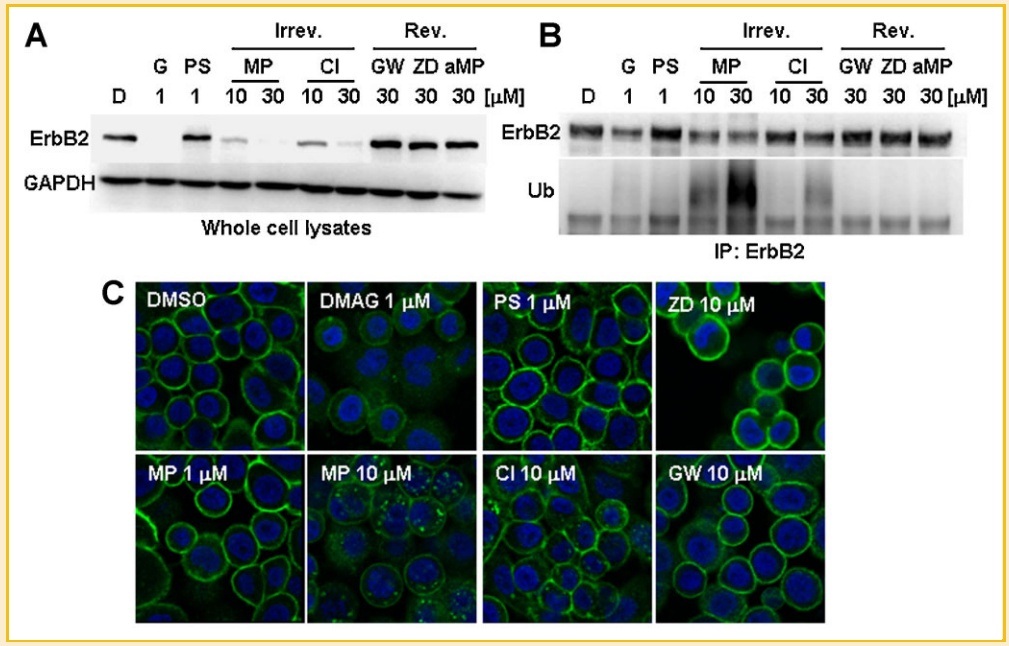


Fig. 2. Irreversible tyrosine kinase inhibitors (Irrev.), but not reversible inhibitors (Rev.), induce ubiquitination, internalization, and downregulation of ErbB2. A: T-47D cells were treated with DMSO (D), MP-412 (MP), CI-1033 (CI), GW-2016 (GW), ZD-1839 (ZD), DMAG (G), PS-341 (PS), or amine-reduced MP-412 (aMP) at the indicated concentrations for 6 h. Cells were lysed, and the protein extracts were analyzed by western blotting with anti-ErbB2 and anti-GAPDH antibodies. B: SK-BR-3 cells were treated with the compounds for 2 h, ErbB2 proteins were immunoprecipitated, and analyzed by western blotting with anti-ErbB2 and anti-ubiquitin antibodies. C: Confocal immunofluorescence images stained for ErbB2 (green) with the Hoechst 33342 nuclear marker (blue) of SK-BR-3 cells treated with the compounds for 6 h.

internalization, and degradation of ErbB2. Furthermore, these activities of MP-412 was more noticeable than CI-1033.

### MP-412-MEDIATED UBIQUITINATION AND DEPLETION OF ErbB2 REQUIRES NEW PROTEIN SYNTHESIS

To examine the mechanism of ErbB2 depletion by MP-412, SK-BR-3 cells were treated with cycloheximide, PS-341 or $NH_4Cl$ (lysosomal inhibitor) prior to adding MP-412, and we observed that only cycloheximide prevented MP-412-induced ubiquitination and depletion of ErbB2 (Fig. 3A). Moreover, in confocal immunofluorescence imaging using anti-ErbB2 antibody, cycloheximide substantially prevented the appearance of intracellular ErbB2-aggregates induced by MP-412 and retained the cell surface expression of the receptor, whereas pretreatment with PS-341 markedly diminished the ErbB2 immunoreactivity in both cell surface and cytosol suggesting that ErbB2 degradation was rather augmented by proteasome inhibition (Fig. 3B). Furthermore, PS-341 increased MP-412-mediated accumulation of ErbB2 in the NP-40 insoluble fraction, which was reminiscent of the observation when the proteasome and Hsp90 chaperone were inhibited simultaneously (Fig. 3C). The lysosomal inhibitor $NH_4Cl$ did not prevent MP-412-mediated ubiquitination and depletion of ErbB2 (Fig. 3A and C). These results suggest that (1) newly synthesized proteins or those with a short turnover may be involved in the MP-412-induced ubiquitination and internalization of ErbB2; and (2) several pathways including proteasome and lysosome are possibly involved in MP-412-mediated ErbB2 depletion while detailed mechanism needs to be determined in the future.

### MP-412 INDUCES Hsp90-RELATED CELLULAR STRESS RESPONSE AND DEPLETES ER-α

We hypothesized that MP-412 would affect the expression level of Hsp90 client proteins besides ErbB2 as well as Hsp90-related cellular stress responses. Both ER-α and Raf-1 are Hsp90 client proteins expressed in breast cancer cells, and Hsp70 is a well-known biomarker of 17-AAG according to clinical trials with cancer patients [Banerji et al., 2005]. In western blotting, MP-412 decreased ErbB2, ER-α, and to some extent, affected Raf-1 in MCF7 cells, indicating that the action of MP-412 is not ErbB2 specific (Fig. 4A). Moreover, like DMAG, MP-412 induced the expression of Hsp70 (Fig. 4A). However, MP-412 was less potent than DMAG in downregulating these client proteins (Fig. 4A and B). Fig. 4C demonstrates that pretreatment with PS-341 noticeably increased MP-412-mediated accumulation of ER-α and ErbB2 in the NP-40 insoluble fraction suggesting that ubiquitin conjugated proteins were aggregated within insoluble compartment to be presumably fated to the degradation. In contrast, pretreatment with cycloheximide canceled MP-412-induced degradation of ER-α and ErbB2 in NP-40 soluble fraction. To the best of our knowledge, this is the first published report demonstrating that an anilinoquinazoline class of kinase inhibitor downregulates estrogen receptor which is one of the most important molecules for breast cancer therapy. These results suggest that MP-412 exhibits some aspects of cellular stress response similarly seen with existing Hsp90 inhibitors.

### MODULATION OF Hsp90 FUNCTION BY MP-412

Next, we evaluated the impact of the effect of MP-412 on the function of Hsp90. ErbB2 and ER-α were immunoprecipitated from

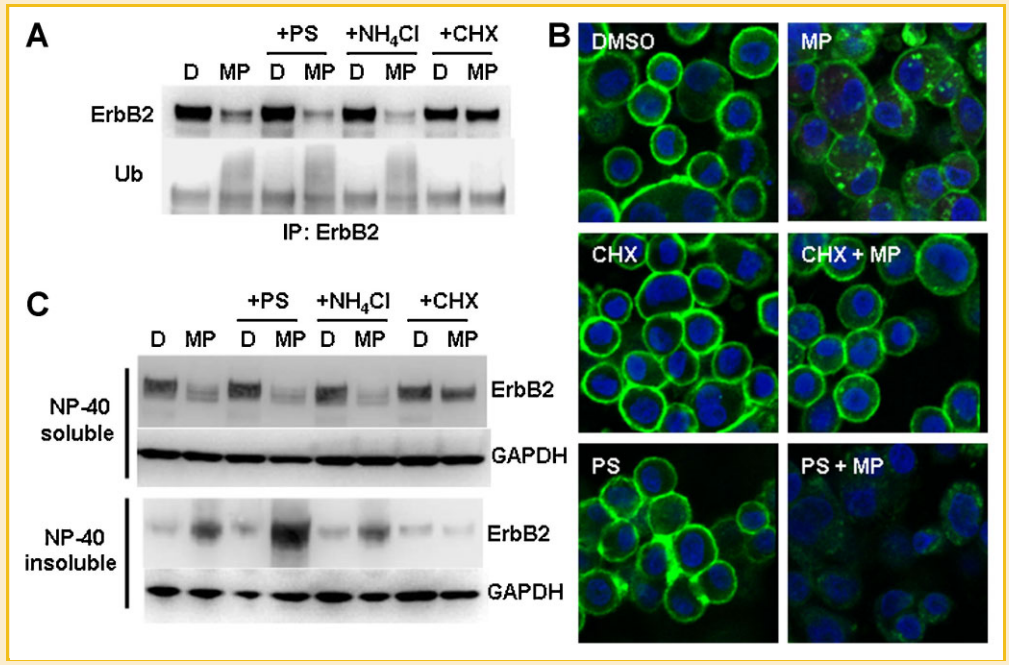


Fig. 3. Ubiquitination and downregulation of ErbB2 induced by MP-412 requires new protein synthesis in SK-BR-3 cells. Cells were pretreated with PS-341 (PS, 1 μM), $NH_4Cl$ (10 mM), or cycloheximide (CHX, 100 μM) for 30 min, followed by treatment with DMSO (D) or MP-412 (MP, 10 μM) for 2 h (A) or 6 h (B and C). A: ErbB2 proteins were immunoprecipitated, and analyzed by western blotting with anti-ErbB2 and anti-ubiquitin antibodies. B: Confocal immunofluorescence images stained for ErbB2 (green) with the Hoechst 33342 nuclear marker (blue). C: Cell extracts were prepared by resuspending in lysis buffer (NP-40 soluble), and the insoluble proteins were treated with sonication in SDS sample buffer (NP-40 insoluble). Protein extracts were immunoblotted with anti-ErbB2 and anti-GAPDH antibodies.

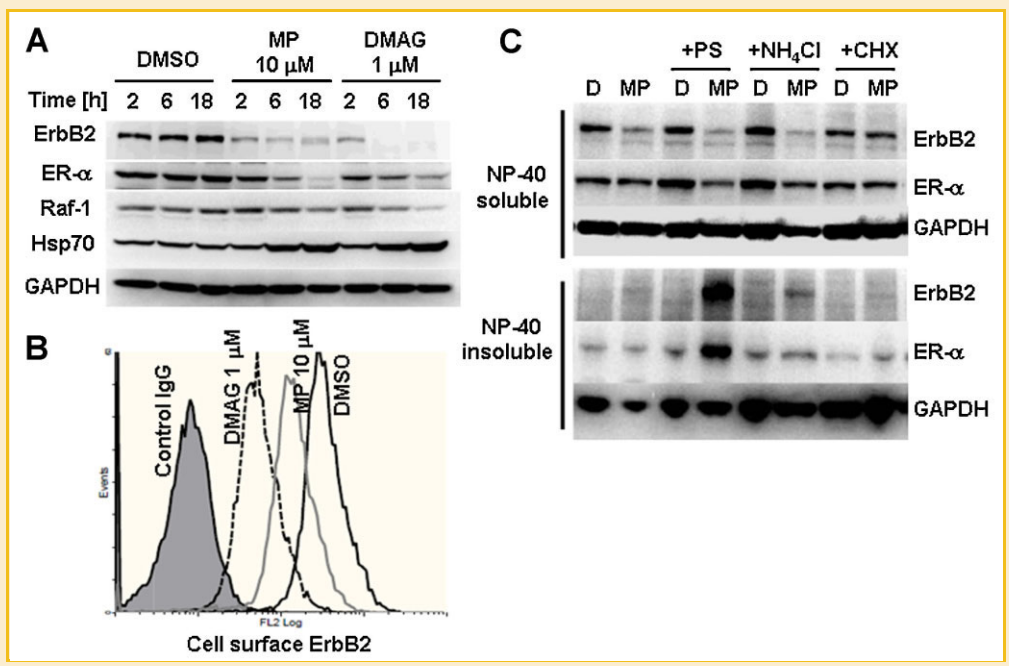


Fig. 4. MP-412 downregulates ErbB2 and ER-α, and upregulates Hsp70 in MCF7 cells. A: Cells were treated with DMSO, MP-412 (MP), or DMAG for the indicated time periods. Protein extracts were analyzed by western blotting with the indicated antibodies. B: Cells were treated with DMSO, MP-412, or DMAG for 6 h. The cell surface expression of ErbB2 was determined by flow cytometry. C: Cells were pretreated with PS-341 (PS, 1 μM), $NH_4Cl$ (10 mM), or cycloheximide (CHX, 100 μM) for 30 min, followed by treatment with DMSO (D) or MP-412 (MP, 10 μM) for 6 h. Cell extracts were prepared by resuspending in lysis buffer (NP-40 soluble), and the insoluble proteins were treated with sonication in SDS sample buffer (NP-40 insoluble). Protein extracts were immunoblotted with indicated antibodies.

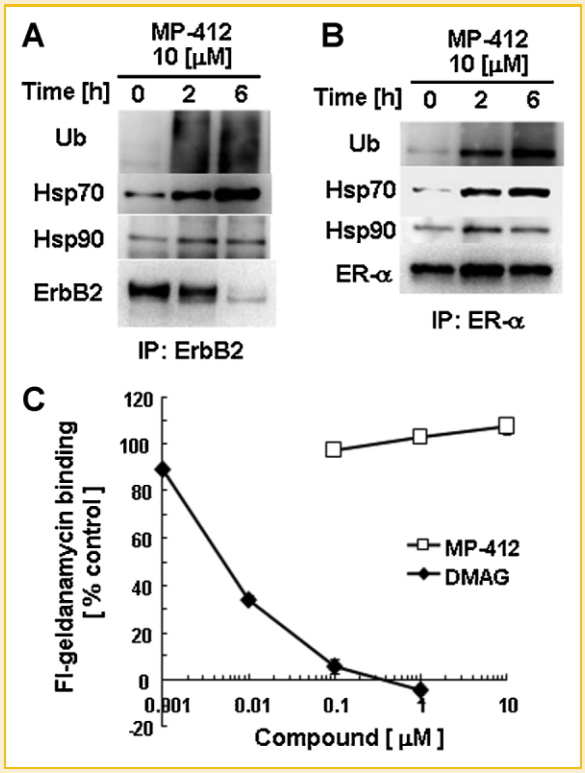


Fig. 5. MP-412 increases Hsp70 chaperone complex with ErbB2 or ER-α but does not show competitive binding to Hsp90. A: SK-BR-3 cells were treated with 10 μM of MP-412 for the indicated time periods. ErbB2 proteins were immunoprecipitated, and analyzed by western blotting with indicated antibodies. B: MCF7 cells were treated with MP-412 as in A, and immunoprecipitated ER-α was analyzed by western blotting with indicated antibodies. C: Increasing concentrations of MP-412 or DMAG were added to a mixture of recombinant human Hsp90-α and fluorescence-labeled geldanamycin, and the mixtures were allowed to incubate for 2 h at room temperature. Specific binding was measured by spectrofluorometry. Data are represented as mean ± SEM of triplicate wells, and are shown as the percentage of labeled geldanamycin binding in the absence of a competitor.

the cell lysates of SK-BR-3 and MCF7 cells, respectively, and the binding of Hsp90 versus Hsp70 to each client protein was analyzed. With concomitant increase of ubiquitination of ErbB2 and ER-α, there was an increase in the amount of Hsp70 binding to them, while Hsp90 binding remained unchanged (Fig. 5A and B). A former report has shown that a shift of multichaperone complex from Hsp90-bound form to Hsp70-bound form increases polyubiquitination of the client proteins to accumulate in the detergent-insoluble cellular fraction directing them to the proteasomal degradation [Citri et al., 2002]. Next, we examined whether MP-412 could act directly on Hsp90 by using a competitive binding assay with fluorescence-labeled geldanamycin and recombinant human Hsp90-α. MP-412 did not compete with the tagged geldanamycin up to 10 μM, while DMAG exhibited 94% inhibition at 0.1 μM (Fig. 5C). Therefore, despite the fact that MP-412 has the ability to destabilize two Hsp90 client proteins and increase Hsp70-complex, it was unlikely that the mechanism of action of MP-412 was through the direct inhibition of Hsp90, and further analysis is required to identify the site of its action.

## DISCUSSION

Our data demonstrated that in addition to kinase inhibition, MP-412 induced ubiquitination, internalization, and degradation of ErbB2 in several human breast cancer cell lines. Moreover, its covalent bonding functionality is likely to be responsible for these biological activities because an amine-substituted MP-412 as well as the reversible inhibitors GW-2016 and ZD-1839 lacked the ability to deplete ErbB2. The results of our study were consistent with the previous report by Citri et al. showing that CI-1033, but not its reversible analog, enhanced the ubiquitination, internalization, and degradation of ErbB2 [Citri et al., 2002]. The other example is a pigment extracted from turmeric, curcumin, which has also been reported to induce ubiquitination and degradation of ErbB2 through the mechanism of forming a Michael adduct with the receptor [Jung et al., 2007]. Here, we further extend the study of Citri et al. [Citri et al., 2002] by demonstrating that MP-412 downregulated not only ErbB2 but also the other Hsp90 client proteins, ER-α and Raf-1 indicating that the action of MP-412 is not ErbB2 specific. Therefore, our findings raise the intriguing possibility that MP-412 exhibits antitumor activity against breast cancer through simultaneous inhibition of ErbB2 kinase and estrogen receptor. In support of this possibility, it has been documented that there is a cross-talk between ErbB2 pathway and estrogen receptor in promoting breast cancer survival and growth [Kurokawa et al., 2000].

Although MP-412 increased Hsp70 in whole cell lysates and also in assembled chaperone complex with ErbB2 and ER-α presumably leading to destabilization of these proteins, the compound did not inhibit directly on the Hsp90 ATP-binding pocket in the cell-free assay. However, it is possible that MP-412 affects indirectly the Hsp90 function by disrupting the interaction between Hsp90 and its cochaperone or by other reasons. One precedent is the triterpenoid derivative celastrol, which impairs Hsp90 function by disrupting the interaction between Hsp90 and its co-chaperone p23 or cdc37 [Hieronymus et al., 2006; Zhang et al., 2008]. Notably, a recent report suggests that celastrol acts as an irreversible inhibitor by forming a Michael adduct using its quinone moiety [Sreeramulus et al., 2009]. Furthermore, celastrol has also been shown to accumulate ubiquitinated proteins and upregulate Hsp70 levels [Westerheide et al., 2004; Yang et al., 2006]. Like other members of the steroid receptor superfamily, unstimulated ER-α exists as multi-protein chaperone complex including Hsp90, and especially p23 cochaperone has been shown to play a key role for not only the stabilization but also the activation of the hormone receptor complex [Morimoto, 2002]. The other example is the histone deacetylase inhibitor LAQ824, which induces acetylation of Hsp90 to inhibit the chaperone association with ErbB2 as well as Raf-1 in breast cancer cells, leading to the proteasomal degradation of these client proteins by shifting the associated chaperone from Hsp90 to Hsp70, which is responsible for the fate directing the destruction of the client proteins [Fujino et al., 2003]. The mode by which MP-412 exhibits Hsp90 inhibition is currently unknown. Therefore, future work may address the mechanistic details of this Hsp90 modulation.

We have herein shown that new protein synthesis is involved in the process of ubiquitination and degradation of ErbB2 and ER-α following MP-412 treatment because cycloheximide blocked this

effect. Moreover, MP-412-mediated upregulation of Hsp70 was also prevented by pretreatment with cycloheximide (data not shown). Hsp70 is a known pharmacodynamic marker of Hsp90 inhibition [Banerji et al., 2005]. Thus, it is possible that the newly synthesized Hsp70 stimulated by MP-412 may be involved in directing ErbB2 and ER-α to proteolysis. In contrast, pretreatment with neither PS-341 nor $NH_4Cl$ prevented degradation of ErbB2 and ER-α mediated by MP-412, suggesting the involvement of more than one pathway beneath the degradation system of these proteins. One of the major pathways for proteasome-independent degradation of ubiquitinated proteins is the lysosomal pathway [Mukhopadhyay and Riezman, 2007]. Further studies will be needed to elucidate the pathway implicated in MP-412-mediated protein degradation.

When considering the new protein synthesis involved in MP-412-induced ubiquitination, we hypothesized that a certain enzyme among ubiquitin system may mediate the activity of MP-412. In particular, the E3 ubiquitin ligase has a crucial role in the elongation of polyubiquitin chains and their transfer onto a specific substrate; notably, there are approximately 1000 E3 ubiquitin ligases in mammals [Sato et al., 2008]. Recently, it was reported that the chaperone-dependent ubiquitin ligase, carboxy terminus of Hsc70-interacting protein (CHIP), is involved in geldanamycin-induced ErbB2 degradation [Zhou et al., 2003]. In the cases of curcumin, quercetin, and even CI-1033, drug-induced ubiquitination and downregulation of ErbB2 has been demonstrated to be mediated by the CHIP protein [Citri et al., 2002; Jung et al., 2007; Jeong et al., 2008]. However, the degradation of ErbB2 by geldanamycin treatment occurs even in $CHIP^{-/-}$ cells, suggesting that CHIP is not the only E3 ubiquitin ligase mediating the activity of geldanamycin [Xu et al., 2002]. Most recently, Ehrlich et al. demonstrated that the Cullin5-RING E3 ubiquitin ligase is recruited to the Hsp90 complex with ErbB2 after treatment with geldanamycin; this further induces polyubiquitination and proteasomal degradation of ErbB2, suggesting the importance of other E3 ligases [Ehrlich et al., 2009]. Both CHIP and Cullin have been reported as the ubiquitin ligase for ER-α as well in breast cancer cells [Fan et al., 2003, 2005]. Therefore, it is interesting to determine whether MP-412 induces protein synthesis of E3 ligases to enhance protein ubiquitination. One more molecule affecting ErbB2 degradation is the prolyl isomerase Pin1, which has been reported to be highly expressed in ErbB2-positive breast cancers and regulates stabilization of ErbB2 by influencing the processes following ubiquitination [Lam et al., 2008].

Finally, degradation of both ErbB2 and ER-α by accelerating the ubiquitin-proteolysis system will become an attractive approach for breast cancer therapy. Although MP-412 was not originally designed to act on the ubiquitin-proteolysis system, further improvement of the effectiveness of such compounds by enhancing the structure-activity relationship will enable development of more potent agents effective for ErbB2- and ER-α-expressing breast cancer.

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