

Geldanamycin destabilizes HER2 tyrosine kinase and suppresses Wnt/ β -catenin signaling in HER2 overexpressing human breast cancer cells

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Abstract. HER2 (also known as ErbB2) is a transmembrane tyrosine kinase whose surface overexpression is linked to tumorigenesis and poor prognosis in breast cancer patients. β -catenin is a substrate of this kinase, and HER2-dependent phosphorylation of tyrosine 654 leads to dissociation of the E-cadherin- β -catenin membrane complex and increased Wnt signaling. β -catenin-mediated Wnt signaling promotes proliferation and invasion of breast cancer cells. In this study, we show that HER2 binds to β -catenin and that geldanamycin (GA), a drug that destabilizes HER2 protein, causes rapid depletion of HER2, thereby disrupting its association with β -catenin in SKBr3 human breast cancer cells. Interestingly, GA did not affect the stability of β -catenin protein, but altered its subcellular localization, driving it out of the nucleus and increasing its association with E-cadherin. Importantly, the change in subcellular localization of β -catenin was also associated with a significant decrease in proliferation and motility of GA-treated breast cancer cells. Moreover, GA treatment led to reduced expression of the Wnt signaling target and cell cycle-promoting gene *cyclin D1*, providing a potential mechanism for the reduced proliferation. In conclusion, GA treatment suppressed tumorigenicity in the human breast cancer cell line SKBr3, at least in part through destabilization of the HER2 oncoprotein and repression of the Wnt/ β -catenin signaling pathway. These findings provide evidence for the clinical importance of GA in treatment of HER2 overexpressing breast cancers.

Introduction

HER2 is a member of the epidermal growth factor receptor (EGFR) family that is overexpressed in about 25-30% of breast cancers and, to a lesser degree, in other cancers (1). Survival and time to relapse are shorter in patients whose tumor cells overexpress HER2 (2,3). HER2 overexpression has been shown to enhance proliferation, survival, and possibly metastatic potential (4). Interestingly, HER2 activation in melanoma cells has recently been found to be associated with dysregulation of β -catenin, a central member of the Wnt signaling pathway (5).

β -catenin was originally identified as a component of cell-cell adhesion complexes containing E-cadherin, β -catenin, α -catenin and actin. It has become clear during the past decade that β -catenin also controls transcription, in concert with TCF/LEF proteins, of Wnt target genes including *cyclin D1* and *c-Myc* (6,7). In normal epithelial cells, β -catenin associates with the cytoplasmic domain of E-cadherin, linking this transmembrane adhesion molecule to the actin cytoskeleton via α -catenin. Recently, several researchers have reported that HER2 can interact with the carboxyl terminus of β -catenin near tyrosine-654 (Tyr-654) (8), and the Tyr-654 residue of β -catenin is preferentially phosphorylated under conditions that disrupt β -catenin-E-cadherin association (9). Thus, the phosphorylation status of Tyr-654, a HER2-binding domain, regulates β -catenin association with E-cadherin. When released from E-cadherin, uncomplexed β -catenin is rapidly degraded by cytosolic ubiquitin-dependent proteasomes. However, mutation of certain Wnt signaling pathway components can lead to accumulation of uncomplexed β -catenin that can translocate to the nucleus where it can activate transcription of a number of proliferation-promoting genes (7). Nuclear accumulation of β -catenin is thus thought to play a pivotal role in tumor progression, and may at least partially explain the tumorigenicity of HER2 overexpressing breast cancer cells.

The antibiotic benzoquinone ansamycin (GA) is a novel anticancer agent that inhibits the molecular chaperone Heat shock protein 90 (Hsp90) by occupying its NH₂-terminal ATP-binding site (10,11). The GA derivative, 17-allylamino-geldanamycin (17AGG), is now in Phase I clinical trial at several centers worldwide (12). Previous studies have shown

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Abbreviations: HER2, human epidermal growth factor receptor 2; GA, geldanamycin

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that GA-mediated degradation of HER2 protein can prevent proliferation and invasion of HER2 overexpressing breast cancer cells via several signaling pathways, including AKT and PI3K (13). However, the role of Wnt signaling in HER2 overexpressing breast cancer cells has been only slightly investigated. The human breast cancer cell line SKBr3, which overexpresses HER2, also exhibits aberrant activation of the Wnt signaling pathway (14). In this study, we show that the antitumor activity of GA can be explained by degradation of HER2 and repression of the Wnt signaling pathway, using SKBr3, a human breast cancer cell line.

Materials and methods

Cell culture. The human breast cancer cell line SKBr3 was obtained from American Type Culture Collection and maintained in 1:1 mixture of DMEM and F12 media, supplemented with 3 mM glutamine, 50 U/ml penicillin, 50 U/ml streptomycin and 10% heat-inactivated fetal bovine serum (Hyclone).

Reagents. GA (Sigma) in DMSO (Sigma) (5 mM) was diluted in culture medium to final concentrations of 5, 10 and 20 nM, with final DMSO concentrations not exceeding 0.01%. Antibodies used for immunoprecipitation and immunoblotting studies were as follows: anti-HER2, anti-E-cadherin, anti- β -actin (all from Santa Cruz), anti- β -catenin (Neomarker), and HRP-conjugated goat anti-rabbit (Pierce). All other reagents were from Sigma, unless otherwise specified.

Cell proliferation test by MTT reduction. The cytotoxicity on SKBr3 cell proliferation was quantified by measurement of the reduction of MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to produce a dark blue formazan product. Cells in the exponential phase of growth were harvested and seeded in 96-well plates (Costar) at a density of 1×10^4 cells per well. After 24 h, drugs were added to triplicate wells for each concentration (0, 5, 10 and 20 nM) and incubation for 24 and 48 h, then MTT was added to each well and the plate was incubated at 37°C in a CO₂ incubator. After 4-h incubation, the medium was removed, and 150 μ l DMSO was added to each well to dissolve the formazan crystals and optical density (OD) was measured at a wavelength of 570 nm using a microplate reader (Multiskan MK3). The production of formazan crystals, and therefore the intensity of color after their dissolution, is proportional to the number of viable cells. Proportions of viable cells in the treatment groups were calculated relative to the controls (100%). IC₅₀ is calculated as the drug concentration that inhibits cell growth by 50% compared with control growth.

Immunoprecipitation and immunoblotting. Cells were grown to 60-70% confluence and exposed to drug or DMSO for the indicated time periods. Cells were washed twice with ice-cold PBS and incubated 30 min in ice-cold lysis buffer (20 mM Tris-HCl pH 8.0, 1% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM DTT, 1 mM sodium vanadate, 50 mM NaF, 50 mM, and complete protease inhibitors (Roche, one tablet complete protease inhibitor cocktail in 50 ml lysis buffer), then scraped, collected, and

clarified by centrifugation (14000 x g for 15 min at 4°C). Protein concentrations were determined using a BCA kit (Pierce), according to the manufacturer's instructions. Immunoprecipitations and immunoblotting were performed as described (15), using the Super Signal chemiluminescent substrate (Pierce). Films were scanned, and bands were quantified using image analysis software (NIH Image).

To analyze β -catenin in nuclear fractions, intact nuclei were prepared from exponentially growing cells by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to manufacture's instructions, and complete protease inhibitors (Roche) was added to the reagents. Nuclear extracts of these samples were fractionated by 8% SDS-PAGE, electrotransferred to nitrocellulose membranes, and analyzed for β -catenin by Western blotting.

RT-PCR and real-time PCR. Cells (2×10^6) were harvested, washed, and total-RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNA was synthesized from isolated RNA using the RevertAid™ first strand cDNA synthesis kit (Fermentas). Primers used for cyclin D1 were: AATGACCCCGCACGATTTC and TCAG GTTCAGGCCTTGCAC. The β_2 -microglobulin gene was used as an internal control. Primers used for β_2 -microglobulin were: GAGTATGCCTGCCGTGTG and AATCCAAATGCGGCA TCT. RT-PCR was performed using 1 μ g template, 2 μ l of 1X PCR buffer (Takara), 1.6 μ l of dNTP mixture (Takara), 0.4 μ M of each primer and 0.5 U of Takara Taq (Takara) in a final volume of 20 μ l. PCR conditions were as follows: 95°C for 5 min, 35 cycles: 94°C for 20 sec, 60°C for 45 sec, 1 min at 72°C, then followed by 10 min of a final extension step at 72°C. PCR products (190 and 109 bp, respectively) were analyzed by electrophoresis on a 3% agarose gel. Real-time PCR was performed using the ABI PRISM 7000 SDS thermal cycler and SYBRE Green I Mix (Takara) following the manufacturer's protocol, using the following conditions: 1 cycle at 95°C for 3 min, 45 cycles at 95°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec where fluorescence was measured, this cycling was followed by melting curve analysis to distinguish specificity of the PCR products. Measurements were normalized against β_2 -microglobulin levels.

Flow cytometric cell cycle analysis. Cell cycle distribution was assayed according to Nusse *et al* (16) using a Becton-Dickinson fluorescence-activated cell scanner and analyzed using the CellCycle Multi-cycle System (Phoenix Flow System).

Motility assay. Approximately 5×10^4 cells were seeded onto cell culture inserts (8 μ m pore size, Falcon 3097) in 24-well plates containing 0.7 ml medium. Cells were incubated for 24 or 48 h with increasing concentrations of GA (0, 5, 10 and 20 nM). After GA treatment, the number of cells adhering to the bottom of the well was counted. Motility was defined as the number of adherent cells after GA treatment relative to control, untreated wells. Bound cells were identified for counting by Wright stain.

Statistical analysis. Statistical analyses were performed using SPSS 12.0 for Windows, and the Student's t-test was used to

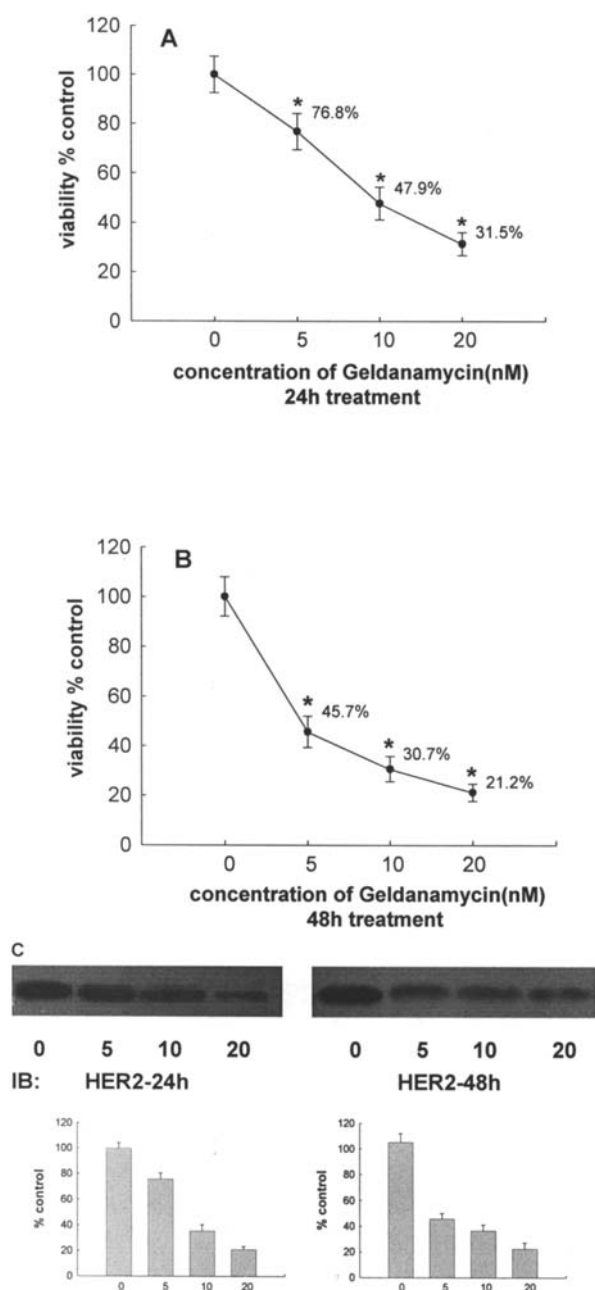


Figure 1. Effect of GA on the viability of SKBr3 cells cultured *in vitro* for 24 and 48 h, and viable cell numbers was determined by MTT assay. Proliferation was evaluated by calculating percentage of viable cells in the treatment groups relative to the controls (100%). Data are the means \pm SD. $n=3$, $^*P<0.05$. Bars, SD. GA inhibited proliferation of SKBr3 cells in a dose-dependent fashion, after 24 h (A) and 48 h (B) treatment. The IC_{50} for each treatment is 9.6 and 4.3 nM, respectively ($n=3$). (C), Immunoblot comparison of HER2 protein expression in cells 24 and 48 h after GA treatment. GA induces a dose-dependent degradation of the HER2 tyrosine kinase ($n=3$). Bars, SD.

compare the differences between treatments and controls. $P<0.05$ was considered to be statistically significant. All other data are presented as mean \pm SD.

Results

The anti-proliferative effects of GA treatment correlate with HER2 levels. GA causes degradation of several signaling proteins. The HER family of kinases are particularly sensitive

to GA-induced degradation, especially HER2 (17). We assessed whether cells that overexpress HER2 are sensitive to GA treatment. We treated the breast cancer cell line SKBr3, which expresses high levels of HER2 protein, with increasing concentrations of GA for 24 and 48 h and evaluated the effects on cell proliferation which was assessed using the MTT assay (18). The concentrations that elicited 50% inhibition of proliferation (IC_{50}) after GA exposure were determined. After 24 h of GA treatment, cell proliferation was inhibited in a dose-dependent fashion with an IC_{50} value of 9.6 nM (Fig. 1A). Interestingly, after 48 h of treatment, GA continued to repress cell proliferation in a dose-dependent manner, but the IC_{50} value decreased to 4.3 nM (Fig. 1B), and 5 nM GA repressed proliferation more after 48 h than after 24 h. These results indicate that while 10 nM GA can repress the proliferation of more than 50% of HER2-overexpressing cells, 5 nM GA can repress an equivalent number of cells after 48 h of treatment.

Protein expression levels for HER2 were evaluated by Western blot. As expected, GA induced a dose-dependent degradation of the HER2 tyrosine kinase (Fig. 1C). Densitometric analysis revealed that $>70\%$ of HER2 protein, relative to control, had been degraded by 24 h of treatment with 20 nM GA. However, 5 nM GA required a longer incubation time to destabilize HER2 protein efficiently, consistent with the effects of this dose on cell proliferation.

GA treatment suppresses aberrant activation of the Wnt/ β -catenin signaling pathway and increases binding between β -catenin and E-cadherin. Previous studies have demonstrated a functional relationship between β -catenin and HER2 tyrosine kinase in tumor progression (5,19). β -catenin is a substrate of the HER2 tyrosine kinase. Phosphorylation of Tyr-654 of β -catenin by HER2 leads to dissociation of the E-cadherin- β -catenin adhesion complex and activation of the Wnt signaling pathway (5,19). We therefore examined whether the interaction between HER2 and β -catenin could be detected in SKBr3 breast cancer cells. Cells were lysed in lysis buffer and immunoprecipitated with an antibody specific for HER2, then examined by SDS-PAGE and immunoblotting using a β -catenin-specific antibody (Fig. 2A). The results demonstrated the existence of an interaction between HER2 and β -catenin in SKBr3 breast cancer cells, suggesting that GA-mediated degradation of HER2 could abrogate this interaction.

Translocation of β -catenin from the membrane to the nucleus leads to activation of the Wnt pathway (20). We next asked whether the disruption of function interaction between HER2 and β -catenin, mediated by GA, could change subcellular location of β -catenin and thus influence the Wnt signaling pathway in SKBr3 cells. After GA treatment, total- β -catenin levels were unchanged, relative to β -catenin levels in untreated cells (Fig. 2B). However, the level of nuclear β -catenin decreased markedly following GA treatment (Fig. 2C). This result suggests that β -catenin is transported from the nucleus to the cytoplasm and/or membrane as a consequence of GA treatment, and possibly HER2 degradation. Two lines of evidence suggest that the change in subcellular localization of β -catenin may be due directly to degradation of HER2. First, the change in subcellular localization of β -catenin appears to follow a similar dose-response curve as the degradation of HER2 protein. Second, GA treatment disrupted

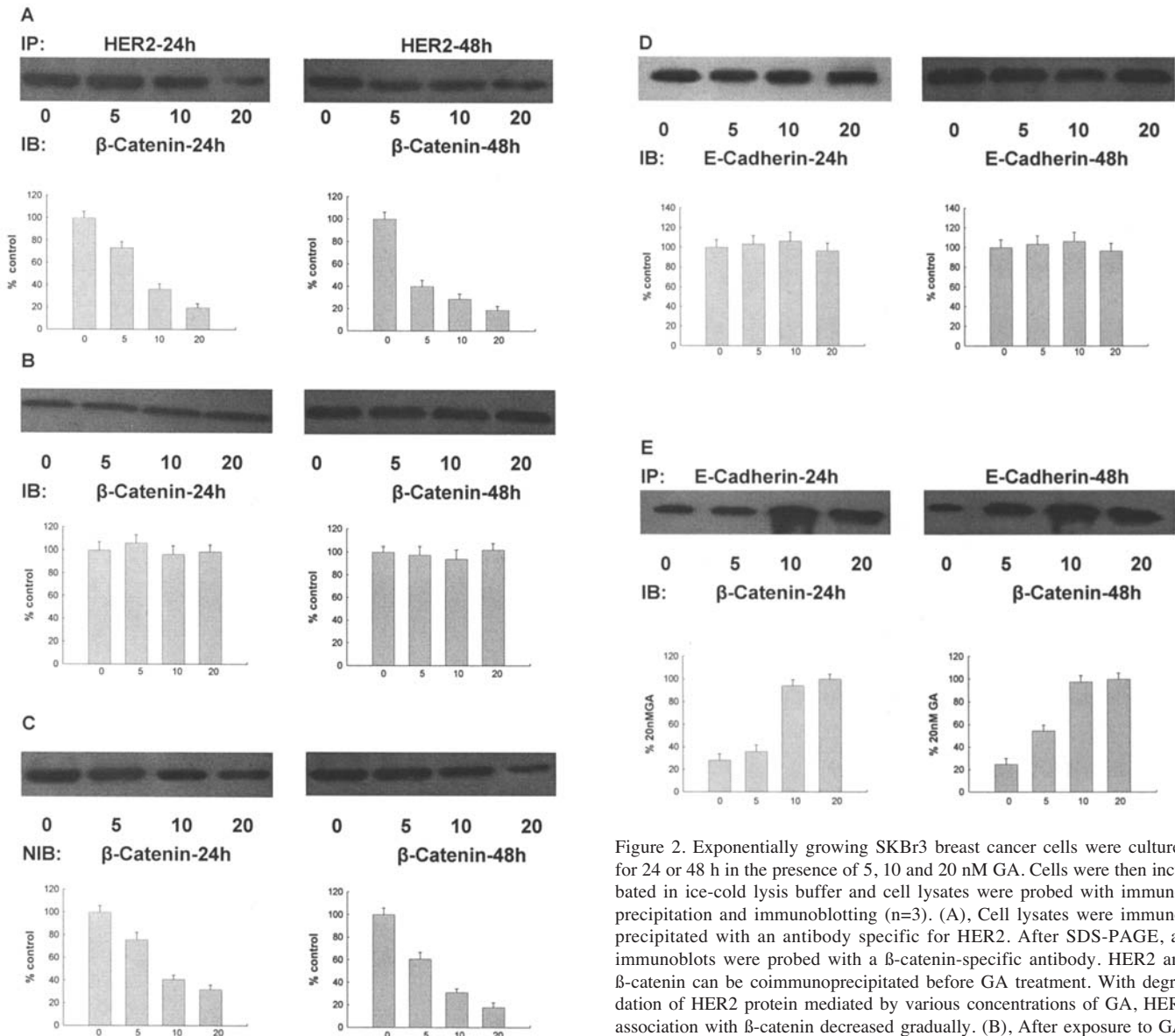


Figure 2. Exponentially growing SKBr3 breast cancer cells were cultured for 24 or 48 h in the presence of 5, 10 and 20 nM GA. Cells were then incubated in ice-cold lysis buffer and cell lysates were probed with immunoprecipitation and immunoblotting (n=3). (A), Cell lysates were immunoprecipitated with an antibody specific for HER2. After SDS-PAGE, all immunoblots were probed with a β -catenin-specific antibody. HER2 and β -catenin can be coimmunoprecipitated before GA treatment. With degradation of HER2 protein mediated by various concentrations of GA, HER2 association with β -catenin decreased gradually. (B), After exposure to GA, the total- β -catenin of immunoblots remains at steady-state level. (C), Nuclear lysates were separated as described in Materials and methods to detect nuclear β -catenin by immunoblotting using anti- β -catenin antibody. The level of nuclear β -catenin declined markedly after efficient GA treatment. (D), After GA treatment, cell lysates were immunoprecipitated with an antibody specific for E-cadherin. After SDS-PAGE, all immunoblots were probed with a β -catenin-specific antibody. GA treatment increased association between E-cadherin and β -catenin. (E), Total-E-cadherin level of immunoblots was not affected by GA treatment. Bars, SD.

binding between HER2 and β -catenin, leading to nuclear export of β -catenin, consistent with reduced Wnt signaling. Our results are therefore consistent with a model in which GA treatment of SKBr3 breast cancer cells leads to degradation of HER2 and repression of the Wnt signaling pathway.

In normal breast epithelial cells, β -catenin associates with the cytoplasmic domain of E-cadherin, near the plasma membrane, and this association is critically important for the maintenance of tight cell-cell contacts (21). To investigate whether the nuclear export of β -catenin enhances its association with E-cadherin, we next examined the amount of β -catenin associated with E-cadherin before and after GA treatment. Cell lysates were immunoprecipitated with an antibody specific for E-cadherin and examined by immunoblotting using a β -catenin-specific antibody and densitometry (Fig. 2D). As expected, the amount of β -catenin coimmunoprecipitated with E-cadherin increased in proportion to the amount that nuclear β -catenin decreased in GA-treated samples, while total-E-cadherin levels remained unchanged (Fig. 2E). Interestingly, in untreated samples, some β -catenin still remained associated with E-cadherin. These data suggest that not all β -catenin is

nuclear in SKBr3 breast cancer cells, and that β -catenin therefore plays a dual-role in this cell line.

GA treatment inhibits β -catenin-dependent transcription in SKBr3 breast cancer cells. Because GA treatment led export of nuclear β -catenin, we next asked whether β -catenin-dependent transcription was repressed following GA treatment. We therefore monitored the effects of GA on the expression of the β -catenin target gene *cyclin D1*, with and without GA treatment, by RT-PCR and real-time PCR. GA treatment inhibited *cyclin D1* expression in a dose-dependent manner, and in a manner that correlated with levels of nuclear β -catenin (Figs. 3 and 2C). Thus GA inhibited transcription of *cyclin D1*, a target of β -catenin-dependent Wnt signaling. Since cyclin D1

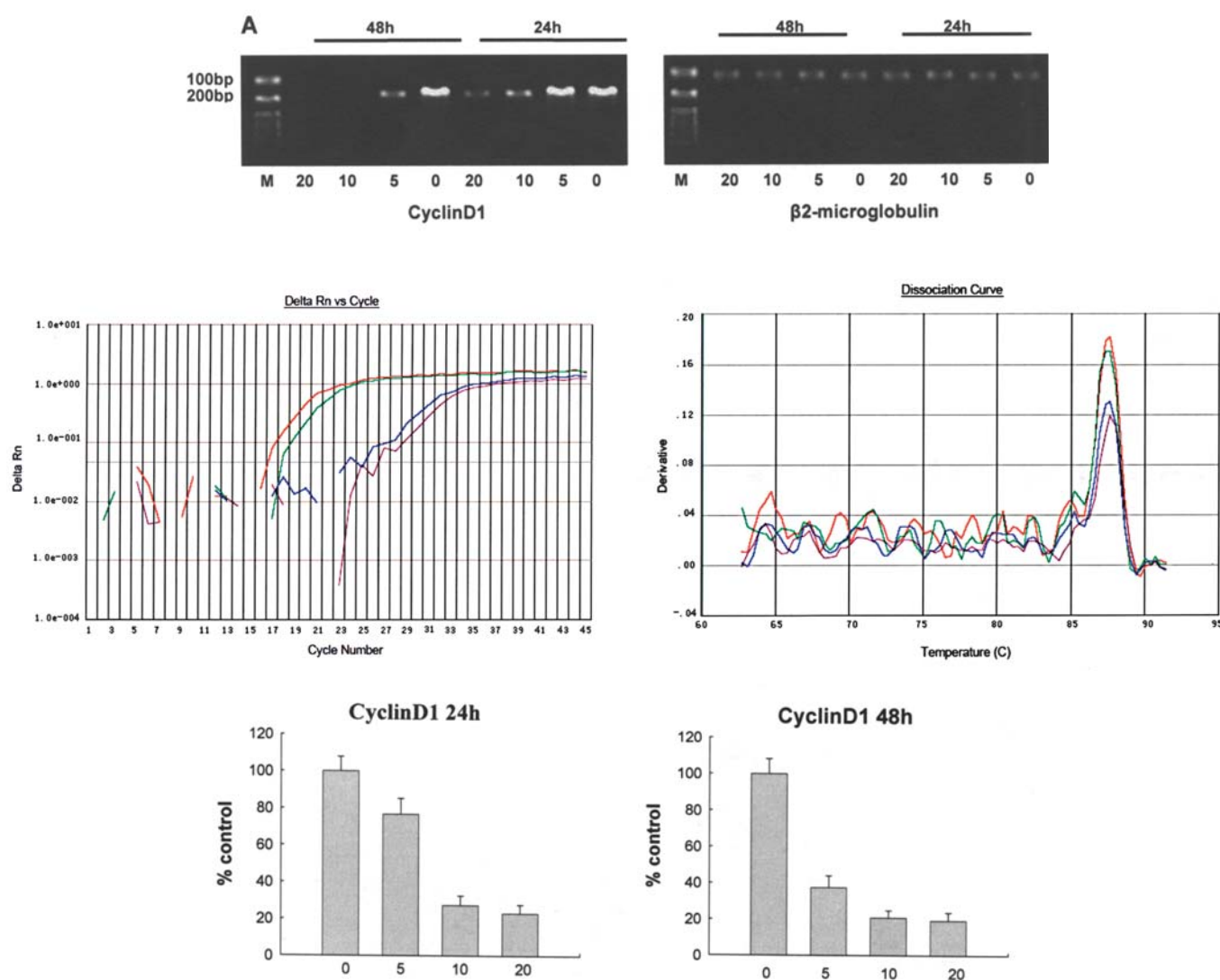


Figure 3. RT-PCR (A) and real-time PCR (B) analysis of *cyclin D1* mRNA and β_2 -microglobulin mRNA after GA treatment (nM). GA markedly reduced transcription of *cyclin D1* in a dose-dependent manner (n=3). Bars, SD. (M, markers; PCR products of *cyclin D1* and β_2 -microglobulin was 190 and 109 bp, respectively).

is an important cell cycle gene, GA-induced reduction of *cyclin D1* levels is consistent with the anti-proliferative effects mentioned above.

GA treatment arrests SKBr3 breast cancer cells in the G1 phase of the cell cycle. G1 cell cycle arrest in response to GA treatment has been observed in cell lines that express the Retinoblastoma (Rb) oncoprotein, while cells lacking Rb arrest in the G2/M phase of the cell cycle (22). Since SKBr3 is an Rb-positive breast cancer cell line (23), we next asked whether GA treatment led to G1 cell cycle arrest using flow cytometric analysis. We found that GA treatment arrested SKBr3 cells in the G1 phase of the cell cycle (Fig. 4). These observations are consistent with reduced levels of *cyclin D1* following GA treatment (Fig. 3) because cyclin D1 is an essential G1 cyclin, involved in regulating the G1-S transition. These data suggest a mechanism whereby HER2 could regulate cell cycle progression by phosphorylating and stabilizing nuclear β -catenin, increasing *cyclin D1* transcription in

cancerous cells. However, in GA-treated cells, HER2 protein is destabilized, leading to increased exit of nuclear β -catenin, decreased *cyclin D1* transcription, and G1 cell cycle arrest.

GA treatment inhibits SKBr3 cell motility. Because the abundance of membrane-associated β -catenin-E-cadherin complexes is inversely related to cell motility (24), we wished to determine whether GA treatment could reduce the motility of SKBr3 breast cancer cells *in vitro*. Interestingly, when added to a 24-h *in vitro* motility assay, 20 nM GA almost completely abolished motility of SKBr3 cells (Fig. 5). These results are consistent with the observed increase in association of β -catenin and E-cadherin following GA treatment.

Discussion

The HER2 oncogene encodes a 185-kDa EGFR-like transmembrane glycoprotein with tyrosine kinase activity. This oncogene is overexpressed in approximately 15-30% of breast

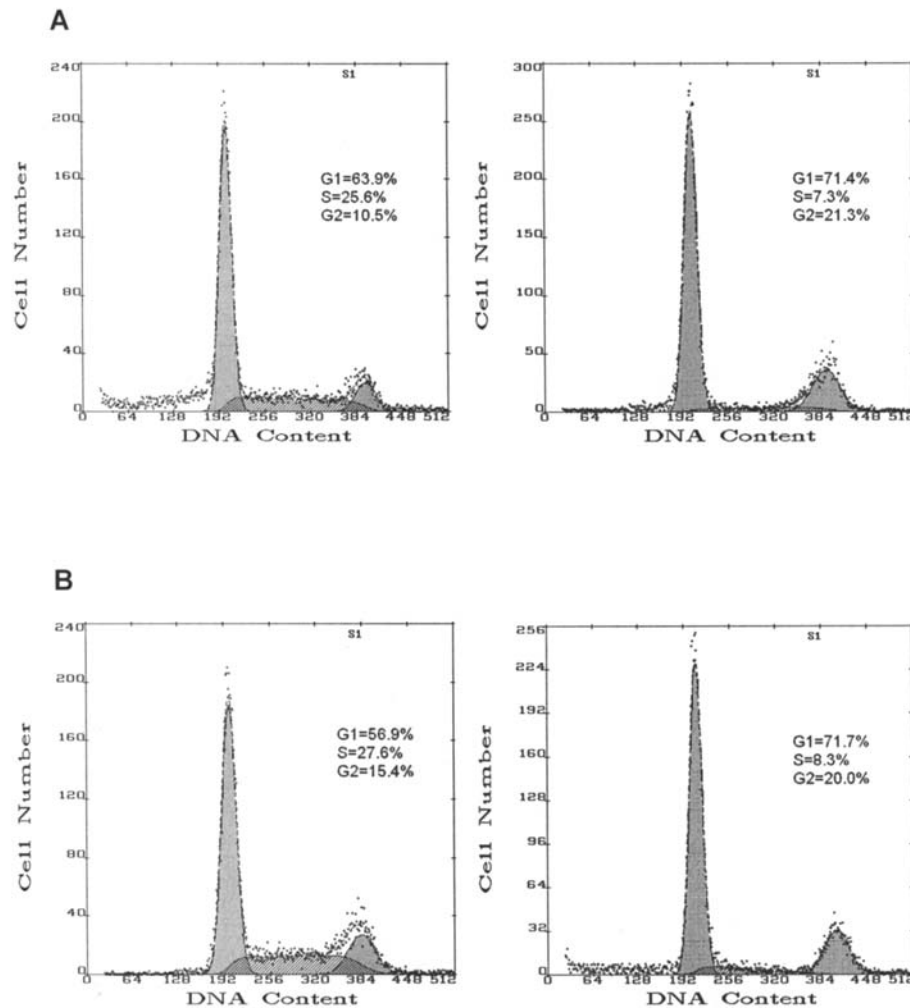


Figure 4. Cell cycle analysis by flow cytometry. The percentage of cells in G1, S and G2 phase is indicated (A) at 24 h and (B) 48 h. GA (5, 10 and 20 nM) treatment causes a dose-dependent G1 block. (A), Before GA treatment, percentage of S phase is 25.6%; after 24-h treatment of 20 nM GA it decreased to 7.3%. (B), Similarly, 48-h treatment of 20 nM GA decreased percentage of S phase from 27.6 to 8.3%.

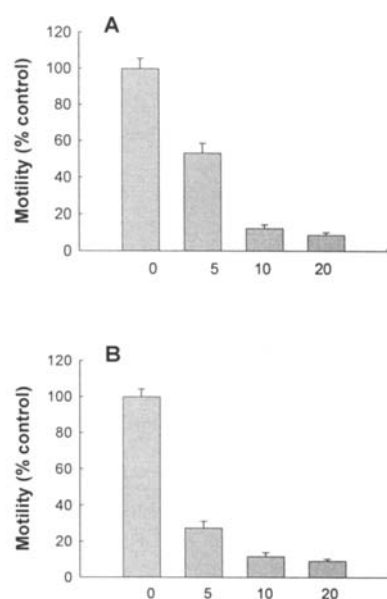


Figure 5. SKBr3 cell motility. GA (nM) markedly reduced the motility of SKBr3 cells (n=3). (A), Motility of SKBr3 cells decreased to 53.3, 12.4 and 8.8% after 24 h of GA treatment (5, 10 and 20 nM). (B), After 48 h of GA treatment, cell motility decreased to 27.5, 11.7 and 9.2% respectively. Bars, SD.

carcinomas, as a result of which cell growth rate is higher and local invasion is increased (25,26). Degradation of HER2 protein following GA treatment has been observed in many human carcinomas, including melanoma, ovarian and breast cancer (5,27,28). In this study we found that GA destabilizes HER2 protein in a dose-dependent manner in a HER2-overexpressing human breast cancer cell line SKBr3. We also found that proliferation was decreased in a dose-dependent manner, consistent with previous studies (5,13, 28-31). Since the biological activity of overexpressed HER2 protein in breast cancer cells remains unclear, interest in identifying a mechanism by which GA-mediated HER2 degradation inhibits breast cancer cell proliferation has received much attention in recent years. Several signaling pathways have been found to be involved in this process, including the phosphatidylinositol 3'-Kinase-AKT-dependent pathway (13,32) and the Wnt/ β -catenin signaling pathway (5). In this study we extend these studies and provide evidence that HER2 degradation-dependent repression of Wnt/ β -catenin signaling can account for some of the anticancer effects of GA treatment on breast cancer cells.

Ectopic activation of Wnt/ β -catenin signaling pathway has been observed in SKBr3 breast cancer cell line (14), so this cell line naturally became the ideal model for research of a

functional relationship between HER2 tyrosine kinase and Wnt/ β -catenin signaling pathway. β -catenin regulates transcription of Wnt signaling targets in concert with TCF/LEF proteins. The transcriptional activity of β -catenin is regulated by its subcellular location (6,7). After GA treatment, the amount of nuclear β -catenin decreased to about 20% of that in untreated cells, while the association between β -catenin and E-cadherin was greatly enhanced. These data suggest that GA treatment and HER2 degradation can switch the activity of β -catenin from transcription to cell-cell adhesion, both of which have implication for tumorigenicity. Decreased nuclear β -catenin leads to decreased expression of the cell cycle-promoting gene *cyclin D1*, whereas increased E-cadherin-associated β -catenin leads to decreased motility. These two key features of tumorigenicity are discussed next.

Cyclin D1 is an essential G1 cyclin, and is involved in regulating the G1-S transition. Overexpression of *cyclin D1* in cultured cells promotes rapid progression from G1 to S phase and increases cell proliferation (33). Cyclin D1 was first shown to be downstream of HER2-mediated transformation in tissue culture models. *In vivo*, *cyclin D1* deficient mice are completely resistant to HER2-mediated mammary tumorigenesis (34). Together, these findings suggest that cyclin D1 is downstream of, and required for, the HER2-mediated tumorigenesis. Cyclin D1 is a major transcriptional target gene of Wnt/ β -catenin signaling pathway, providing evidence for a link between HER2 and Wnt/ β -catenin signaling. Moreover, recent studies have shown that approximately 40% of breast cancers overexpress *cyclin D1* and almost of all of these (92%) also had high levels of active β -catenin (35). These observations strongly suggest that overexpression of *cyclin D1* in HER2-mediated mammary tumorigenesis is a result of ectopic activation of Wnt/ β -catenin signaling pathway. In this study we observed a correlation between HER2 and nuclear β -catenin levels and proliferation, following GA treatment. Moreover, cell cycle analysis showed that GA treatment also leads to a dose-dependent G1 arrest, and that this G1 block is associated with a reduction in *cyclin D1* expression, thereby providing a mechanism by which GA inhibits growth of a HER2-overexpressing breast cancer cell line.

In addition to preventing proliferation, GA treatment may also exert its anti-tumorigenic effects by reducing the motility of SKBr3 cells. The E-cadherin- β -catenin complex plays an essential role in adhesion of normal breast tissue. Disruption of this complex is thought to result in reduced cell-cell adhesion increased invasiveness, without affecting levels of E-cadherin (21). In this study, GA treatment reduced cell motility while increasing levels of the E-cadherin- β -catenin complex. Notably, the amount of E-cadherin remained unchanged before and after GA treatment. These observations strongly support the proposal that GA could reduce the invasiveness of breast cancer cells that, like SKBr3 cells, express high levels of HER2 protein by promoting degradation of HER2 protein and thereby enhancing binding between E-cadherin and β -catenin.

In conclusion, GA reduces the tumorigenicity of the HER2-overexpressing breast cancer cell line SKBr3, at least in part by destabilizing the HER2 oncoprotein, and disruption of the functional relationship between HER2 and the Wnt/ β -catenin signaling pathway. Our findings provide evidence

to support the clinical utility of GA in treatment of HER2 overexpressing breast cancer patients.

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