Tyrosine Kinase Inhibitor Emodin Suppresses Growth of HER-2/neu-overexpressing Breast Cancer Cells in Athymic Mice and Sensitizes These Cells to the Inhibitory Effect of Paclitaxel

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ABSTRACT
Overexpression of the HER-2/neu proto-oncogene, which encodes the tyrosine kinase receptor p185^neu, has been observed in tumors from breast cancer patients. We demonstrated previously that emodin, a tyrosine kinase inhibitor, suppresses tyrosine kinase activity in HER-2/neu-overexpressing breast cancer cells and preferentially represses transformation phenotypes of these cells in vitro. In the present study, we examined whether emodin can inhibit the growth of HER-2/neu-overexpressing tumors in mice and whether emodin can sensitize these tumors to paclitaxel, a commonly used chemotherapeutic agent for breast cancer patients. We found that emodin significantly inhibited tumor growth and prolonged survival in mice bearing HER-2/neu-overexpressing human breast cancer cells. Furthermore, the combination of emodin and paclitaxel synergistically inhibited the anchorage-dependent and -independent growth of HER-2/neu-overexpressing breast cancer cells in vitro and synergistically inhibited tumor growth and prolonged survival in athymic mice bearing s.c. xenografts of human tumor cells expressing high levels of p185^neu. Both immunohistochemical staining and Western blot analysis showed that emodin decreases tyrosine phosphorylation of HER-2/neu in tumor tissue. Taken together, our results suggest that the tyrosine kinase activity of HER-2/neu is required for tumor growth and chemoresistance and that tyrosine kinase inhibitors such as emodin can inhibit the growth of HER-2/neu-overexpressing tumors in mice and also sensitize these tumors to paclitaxel. The results may have important implications in chemotherapy for HER-2/neu-overexpressing breast tumors.

INTRODUCTION
Breast cancer is a major cause of cancer death among women in the United States (1). The HER-2/neu proto-oncogene encodes a M^c^185,000 transmembrane tyrosine kinase receptor (p185^neu). Enhanced expression of HER-2/neu is known to be involved in many human cancers, including breast cancer (2–9), and has been shown to correlate with tumor grade, tumor size, lymph node metastasis, and survival in breast cancer patients (2, 10, 11). Cellular and animal experiments have shown that increases in HER-2/neu tyrosine kinase activity enhance the expression of malignant phenotypes (12–15). In our own experimental system, HER-2/neu overexpression in breast cancer cells was reported recently to induce resistance to a potent new chemotherapeutic drug, paclitaxel (Taxol; Ref. 16), which has been highly effective in treating advanced metastatic breast cancer in clinical trials (17–20). Although controversy exists regarding Taxol resistance induced by HER-2/neu overexpression (see “Discussion”), the association of HER-2/neu overexpression in cancer cells with malignant phenotypes and potential chemoresistance provides a plausible interpretation for the poor clinical outcome for patients with HER-2/neu-overexpressing tumors. Therefore, the HER-2/neu tyrosine kinase receptor may be a useful therapeutic target for HER-2/neu-overexpressing tumors.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), a tyrosine kinase inhibitor (21) isolated from Polygonum cuspidatum, was shown recently to inhibit HER-2/neu tyrosine kinase activity and to preferentially repress the transformation of HER-2/neu-overexpressing breast cancer cells through repression of p185^neu tyrosine kinase, including anchorage-dependent and -independent growth of HER-2/neu-overexpressing breast cancer cells (22). Emodin was also shown to sensitize HER-2/neu-overexpressing lung cancer cells to chemotherapeutic drugs such as cisplatin, doxorubicin, and etoposide in vitro (23). In the present study, we extended our investigations to the in vivo animal system and investigated whether emodin may repress the growth of HER-2/neu-overexpressing breast tumors in mice and whether emodin may sensitize these tumors to taxol.

MATERIALS AND METHODS
Cell Culture and Reagents. The human breast cancer cell lines MDA-MB-361, MDA-MB-453, BT-483, SKBr-3, BT-474, MDA-MB-231, and MCF-7 and the immortalized breast cell line HBL-100 were obtained from American Type Culture Collection (Rockville, MD). MDA-MB-361, MDA-MB-453, BT-483, SKBr-3, and BT-474 cells overexpress HER-2/neu,
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whereas MDA-MB-435, MDA-MB-231, MCF-7, and HBL-100 cells express low levels of HER-2/neu (24). All cells were grown in DMEM/F12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and gentamicin (50 µg/ml). Cells were grown in a humidified incubator at 37°C under 5% CO₂ in air. Emodin and Taxol were purchased from Sigma Chemical Co. (St. Louis, MO) and were dissolved in DMSO before use.

**Proliferation Assay.** Cells were detached by trypsinization, seeded at 2 × 10⁴ cells/ml in a 96-well microtiter plate overnight, treated with different concentrations of either emodin (0, 1, 10, 20, 40, 60, or 80 µM) alone, taxol (0.1, 1, 10, and 100 nM) alone, or emodin (20 and 40 µM) plus taxol (0.1, 1, and 10 nM for MDA-MB-231 and MDA-MB-435 cells, and 0.1, 1, and 10 µM for BT-474 and MDA-MB-361 cells) and equal volume of DMSO as the control, then incubated for an additional 72 h. The effects on cell growth were examined by the MTT assay (25, 26). Briefly, 20 µl of MTT solution (5 mg/ml) were added to each well and was incubated at 37°C for 4 h. The supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 150 µl of DMSO and then monitored with a microplate reader (Dynatech MR 5000 fluorescence; Dynatech Corp., Burlington, MA) at a wavelength of 590 nm.

**Colony Formation in Soft Agarose.** Cells were seeded in 24-well plates (1 × 10⁴ cells/well) in culture medium containing 0.35% agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer with or without either different concentrations of Taxol or emodin alone, or emodin (20 or 40 µM) plus taxol (0.01, 0.1, and 1 nM for MDA-MB-231 and MDA-MB-435 cells; 0.1, 1, and 10 µM for BT-474 and MDA-MB-361 cells) and incubated for 4 weeks at 37°C as described previously (22). Colonies were then stained with p-iodonitrotetrazolium violet (1 mg/ml), and colonies >100 µm were counted. All determinations were made four times.

**Effect of Emodin Alone, Taxol Alone, or Emodin Plus Taxol on Tumor Growth in Mice.** MDA-MB-361 cells (5 × 10⁵) or MDA-MB-231 cells (5 × 10⁶) were injected s.c. into both left and right flanks of female nude mice (four groups, six mice for each group). Three weeks later, when the solid tumors were palpable, the mice were given either placebo, emodin (40 mg/kg body weight), Taxol (10 mg/kg body weight), or emodin (40 mg/kg) plus Taxol (10 mg/kg) by i.p. injection twice a week for 8 weeks. After stopping treatment, one mouse from each group was sacrificed, and tumors were taken out to examine the level of p185<sub>neu</sub> tyrosine phosphorylation in those tumors by immunohistochemical staining and Western blot analysis. The tumor volume was monitored weekly for 8 weeks. The rest of the five mice from each group were observed for survival for 1 year. At the end of the experiment, the remaining mice were sacrificed, and tumors from one mouse in each group were taken out for examining tyrosine phosphorylation and p185<sub>neu</sub> protein with immunohistochemical staining.

**Western Blot Analysis of Level of HER-2/neu Tyrosine Phosphorylation and Expression of p185<sub>neu</sub> Protein in Vivo.** Protein extracts were prepared by homogenizing tumor tissues obtained from control, emodin alone, Taxol alone, and combined emodin and Taxol treated mice with lysis buffer [20 mM Na<sub>2</sub>PO₄ (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM mg/ml leupeptin, 100 mM NaF, and 2 mM Na<sub>3</sub>VO₄]. The protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 50 µg of protein was separated by 6% SDS-PAGE and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing nonfat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). The treated filter paper was incubated with primary antibodies [anti-phosphotyrosine antibody (UBI, Lake Placid, NY) for the detection of phosphotyrosine, anti-p185<sub>neu</sub> antibody c-neu for the detection of p185<sub>neu</sub>, or anti-actin antibody for the detection of actin for equal protein loading]. This was followed by incubation with HRP-goat anti-mouse antibody (1:1000 dilution; Boehringer Mannheim Corp., Indianapolis, IN). Bands were then visualized with an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

**Immunohistochemical Analysis.** Tissue sections were taken from tumor-bearing mice. Frozen sections were fixed with acetone, and the specimens were routinely fixed in formalin and then embedded in paraffin. The sections were subsequently subjected to routine pathological analysis by the modified avidin-biotin complex technique. Tyrosine phosphorylation and expression of HER-2/neu protein were detected by incubating tissue samples with anti-tyrosine phosphorylation antibody (Ab4; Oncogene Science Inc., Monhasset, NY) and rabbit polyclonal antibody against p185<sub>neu</sub> (DAKO Corp., Carpinteria, CA.). The samples were then incubated with biotinylated goat anti-rabbit IgG antibody or horse anti-mouse IgG antibody and finally with avidin-biotin-peroxidase complex (Vector Labs, Inc., Burlingame, CA). Staining was then developed in aminoethyl carbazole chromogen stock solution (Sigma Chemical Co.). Mayer’s hematoxylin was used as a counterstain.

**Evaluation of the Drug Combination.** For evaluating the combined effect of the two drugs, observed values were compared with predicted values (c) calculated from the equation $c = a \times b/100$, where $a$ and $b$ are the survival values with single agents (27, 28). Observed values of <70% of predicted ones were considered synergistic. The interaction of two drugs was also evaluated by a concentration-response isobologram using IC<sub>25</sub> as the reference concentrations.

**RESULTS**

Emodin Enhances the Effects of Taxol on Growth and Transformation of HER-2/neu-overexpressing Human Breast Cancer Cells. To investigate whether the possibility that emodin may sensitize HER-2/neu-overexpressing breast cancer cells to Taxol, we examined the combined effects of emodin and Taxol on growth and transformation of breast cancer cells by using MTT assay and a colony formation assay in soft agarose. To identify optimal conditions for combination

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<sup>3</sup> The abbreviation used is: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
treatment, we first examined the sensitivity of the cells to Taxol. As shown in Table 1, the HER-2/neu-overexpressing breast cancer cells were much more resistant to Taxol than were the breast cancer cells that expressed low levels of HER-2/neu. These results are consistent with the previous report that HER-2/neu overexpression in breast cancer cells induces resistance to Taxol (16).

We then examined the combined effect of emodin and Taxol on the growth of MDA-MB-231 and MDA-MB-435 cells, which express low levels of HER-2/neu, and on MDA-MB-361 and BT-474 cells, which overexpress HER-2/neu (28). We first treated these breast cancer cells with different concentrations of emodin for 72 h, and then we measured the effect of emodin on cell viability. Emodin inhibited cell growth in a dose-dependent manner. IC_{50} of emodin for MDA-MB-361, BT-474, MDA-MB-231, and MDA-MB-435 are 45, 50, 85, and 80 μM, respectively. We used IC_{25} of emodin (20 μM) and taxol as the reference combination dose. Treatment of MDA-MB-361 cells with emodin or Taxol alone inhibited cell growth by only 28 and 16%, respectively. The combination of emodin and Taxol synergistically inhibited cell growth by 70% (Fig. 1c). A similar synergistic effect was observed in the HER-2/neu-overexpressing BT-474 cells (Fig. 1d). When other combinations of emodin (40 μM) and Taxol (0.1, 1 and 10 μM) were analyzed, a synergistic effect was also observed for BT-474 and MDA-MB-361 (data not shown). However, there is no significant synergistic antiproliferative effect in the MDA-MB-231 and MDA-MB-435 cells.

We next examined the combined effect of emodin and Taxol on the transformed phenotypes of MDA-MB-231, MDA-MB-435, MDA-MB-361, and BT-474 cells. Cells were seeded into soft agarose and monitored for colony formation. Treatment of MDA-MB 361 cells with emodin or Taxol alone suppressed colony formation by only 28 and 31%, respectively. The combination of emodin and Taxol synergistically inhibited colony formation by 96% (Fig. 2a). A synergistic effect was also observed in the HER-2/neu-overexpressing BT-474 cells (Fig. 2b). Although additive effects were observed in both MDA-MB-231 and MDA-MB-435 cells which express low levels of HER-2/neu, no such synergistic effect was observed in either MDA-MB-231 or MDA-MB-435 cells (Fig. 2, c and d). The synergistic effect was more profound in the soft agarose assay than in the MTT assay, probably because drug treatment is longer with the soft agarose assay than with the MTT assay (2–3 weeks are required for the soft agarose colony formation assay, whereas only 3 days are needed for the MTT assay). These results indicate that emodin enhances the cytotoxic effect of Taxol on HER-2/neu-overexpressing breast cancer cells and suggest that the tyrosine kinase activity of HER-2/neu is required for the Taxol-resistant phenotype of HER-2/neu-overexpressing human breast cancer cells. Therefore, tyrosine kinase inhibitors such as emodin may improve the efficacy of Taxol in the treatment of HER-2/neu-overexpressing breast cancer cells.

Emodin Preferentially Represses the Growth of HER-2/neu-overexpressing Human Breast Tumors in Athymic Mice. Because emodin preferentially inhibits the transformed phenotype of HER-2/neu-overexpressing breast cancer cell lines (22) and acts synergistically with Taxol to inhibit the growth of these cells in vitro (Figs. 1 and 2), we investigated whether emodin also preferentially suppresses the growth of HER-2/neu-overexpressing tumors in athymic BALB/C nude mice and whether emodin can enhance the inhibitory effect of Taxol on the growth of such tumors.

We induced tumors by injecting MDA-MB-231 cells (5 \times 10^6 cells) or MDA-MB-361 cells (5 \times 10^7 cells) s.c. into both left and right flanks of nude nude female mice. The difference in number of cells injected is due to different growth rates. The doubling time for MDA-MB-361 cells is slower than MDA-MB-231 cells (40 and 24 h, respectively). These doses were sufficient to produce tumors in all of the mice (five mice for each group). Three weeks later, when the solid tumors became palpable, the mice were treated with either placebo (cremophor: DMSO:saline, 1:2:7) or emodin 40 mg/kg of body weight (0.2 ml/mouse) given by i.p. injection twice a week for 8 weeks. The chosen 40 mg/kg dose was based on our initial pilot experiment that a similar dose, 37 mg/kg body weight, is highly effective in suppressing the tumor growth for tumors induced by the activated p185^{neu}-transformed 3T3 cells (twice per week injection for 3 weeks would result in reduction of tumor volume to 37% of the control). Survival was calculated in days from the day cancer cells were injected. Animals were sacrificed when tumors increased to 2 cm in any dimension, as required by the institution’s Animal Care Committee. Mice were observed for survival up to 12 months. All mice with MDA-MB-361 tumors treated with placebo continued to develop tumors (Fig. 3a) and eventually died of tumors between 80 and 167 days (Fig. 4a). Emodin treatment of mice with MDA-MB-361 tumors significantly inhibited tumor growth (P < 0.05; Fig. 3a) and prolonged survival (P < 0.05; Fig. 4a). One mouse lived up to 1 year. However, emodin did not significantly inhibit the growth of MDA-MB-231 tumors (P > 0.3; Fig. 3b), nor prolonged survival of mice with such tumors compared with the placebo (P > 0.4; Fig. 4b). These results indicate that emodin preferentially suppresses the growth of HER-2/neu-overexpressing human breast tumors and prolongs the survival of mice with such tumors.

Emodin Sensitizes HER-2/neu-overexpressing Tumors in Nude Mice to Taxol. Emodin acts synergistically with taxol to inhibit the growth of HER-2/neu-overexpressing human breast cancer cells in vitro (Figs. 1 and 2); we therefore examined whether emodin sensitizes HER-2/neu-overexpressing tumors in athymic nude mice to Taxol. MDA-MB-361 cells were...
injected s.c. into athymic BALB/c nude mice (six mice for each group, two tumors/mouse). Three weeks later, when the solid tumors became palpable, mice were treated with either placebo (cremophor:DMSO:saline, 1:2:7), emodin alone (40 mg/kg of body weight), Taxol alone (10 mg/kg of body weight), or a combination of emodin and Taxol (0.2 ml/mouse) given by i.p. injection twice a week for 8 weeks. The initial tumor sizes for each group were as follows: placebo, 112 ± 692; emodin, 171 ± 140; Taxol, 191 ± 83; emodin and Taxol, 292 ± 182 mm³. After stopping the treatment, one mouse from each group was sacrificed, and tumors from these mice were taken out to examine for the level of p185neu tyrosine phosphorylation by immunohistochemical staining and Western blot analysis. The rest of the mice (five from each group) were observed for survival up to 12 months. At the end of the experiments, we sacrificed the remaining mice and examined the tyrosine phosphorylation as well as p185neu protein in those tumors.

As shown in Fig. 5, all mice treated with placebo continued to develop tumors and eventually died of cancer by day 167. Treatment of the tumor-bearing mice with either emodin alone or Taxol alone inhibited tumor growth (P < 0.05 and P < 0.03, respectively; Fig. 5a) and prolonged survival (P < 0.05 and P < 0.05, respectively; Fig. 5b). However, the inhibitory effect on tumor growth was greatly enhanced by injecting emodin followed by Taxol (P < 0.001; Fig. 5a). The combination treatment was significantly better than either of the treatments alone; only 40% of the mice treated with emodin plus Taxol continued to develop. For the rest of the mice (60% of the mice), the tumors gradually shrank, and no tumors were observed by day 240 (P < 0.001; Fig. 5b). These results indicate that emodin sensitizes the effect of Taxol on suppression of HER-2/neu-overexpressing human breast tumors growth and prolongation of mice survival with such tumors.

Emodin Inhibits Tyrosine Phosphorylation in Vivo. To investigate whether repression of tyrosine phosphorylation of HER-2/neu is connected with the therapeutic effects of emodin on tumors in vivo, tyrosine phosphorylation of HER-2/neu in tumors from the mice in each group (control, emodin alone, Taxol alone, or emodin plus Taxol) was analyzed by immunohistochemical staining (Fig. 6). The control tumor had very strongly positive red staining, which represents tyrosine phosphorylation, but no such staining was detected in emodin-treated tumor. The staining for p185neu expression in emodin-treated tumor was not significantly different from the staining in the
control tumor by counting 10 different microscopic fields. A strongly positive red phosphotyrosine staining was also observed in Taxol-treated tumors but not in combined emodin and Taxol-treated tumors (data not shown). These results were further confirmed by Western blot analysis. Tyrosine phosphorylation levels of HER-2/neu in the emodin-treated tumors obtained at the completion of emodin treatment were barely detectable, compared with levels in the control tumor; however, p185neu protein levels in the emodin-treated tumor were not significantly changed (Fig. 7). These results are consistent with the concept that emodin suppresses the growth of HER-2/neu-overexpressing tumors in nude mice by inhibiting phosphorylation of HER-2/neu tyrosine kinase.

**DISCUSSION**

In a previous study, we demonstrated that emodin suppresses tyrosine kinase activity in HER-2/neu-overexpressing breast cancer cells and preferentially inhibits growth and transformation of these cells in vitro (22). In the present study, we further extended the observation to the in vivo animal system by showing that emodin can inhibit the growth of HER-2/neu-overexpressing breast cancer cells in nude mice. We also demonstrated that emodin is able to sensitize MDA-MB-361 and BT-474 breast cancer cells, which overexpress HER-2/neu to the anticancer drug Taxol in vitro but does not have the same effect in the MDA-MB-231 and MDA-MB-435 cell lines, which express low levels of HER-2/neu. These results suggest that the tyrosine kinase activity of HER-2/neu is required for cell growth and Taxol resistance; and that tumor repression by emodin alone and the synergistic effect of emodin plus Taxol on tumor growth in mice may be due to decreasing tyrosine phosphorylation of p185neu. In the mice with HER-2/neu-overexpressing MDA-MB-361 tumors, emodin significantly reduced tumor growth and prolonged survival. Western blot analysis indicated that
tyrosine phosphorylation of HER-2/neu was significantly decreased by emodin treatment compared with placebo treatment (Fig. 7), and no positive staining for tyrosine phosphorylation was detectable in the emodin-treated tumor (Fig. 6). These results indicated that emodin indeed functions as a tyrosine kinase inhibitor in vivo. Although we cannot rule out the possibility that another tyrosine kinase may also be inhibited by emodin, which could contribute to the biological effects we observed in the animal experiments, we believe that repression of tyrosine kinase activity of p185T is likely the major mechanism accounting for the observed biological effects because the overexpressed p185^{neo} tyrosine kinase in MDA-MB-361 cells (more than one million molecules/cell) is probably the major tyrosine kinase in the cancer cells and has been shown to be required for malignancy (29). In our preliminary study, we found that emodin can also repress epidermal growth factor-induced tyrosine phosphorylation of epidermal growth factor receptor at high concentrations, compared with the concentration which is used to repress tyrosine phosphorylation of HER-2/neu (data not shown). It would be interesting to further study whether emodin might have differential selectivity in repression of tumor activities of different tyrosine kinase molecules in vivo experiments. It is worth mentioning that there was a growth spurt after both MDA-MB-361 and MDA-MB-231 xenografts until about days 15–18. Then, the xenografts began to repress. We also found the similar phenomenon in the other independent in vivo experiments (data not shown). The cause this biphasic nature of growth in both MDA-MB-361 and MDA-MB-231 xenografts in not clear yet.

We and others have shown that the point-mutated rat neu oncogene (neuT) has high tyrosine kinase activity and increases

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**Fig. 3** Effect of emodin on tumor growth in mice bearing MDA-MB-361 (a) or MDA-MB-231 (b) tumors. Either MDA-MB-361 cells ($5 \times 10^7$) or MDA-MB-231 cells ($5 \times 10^6$) were injected s.c. into female nu/nu mice (five mice for each group, 2 sites for each mouse). Three weeks later, when solid tumors were palpable tumor size at the initiation of the treatment as shown at day 0 (a and b), the mice were given either placebo or emodin (40 mg/kg body weight) by i.p. injection twice a week for 8 weeks. The tumor volume was monitored weekly for 8 weeks. Statistical determinations were performed using t test analysis.

**Fig. 4** Effect of emodin on survival times of mice with MDA-MB-361 (a) and MDA-MB-231 (b) tumors. Either MDA-MB-361 cells ($5 \times 10^7$) or MDA-MB-231 cells ($5 \times 10^6$) were injected s.c. into female nu/nu mice (five mice for each group). Three weeks later, when the solid tumors were palpable, the mice were given either placebo or emodin (40 mg/kg body weight) by i.p. twice a week for 8 weeks. Mice were observed for survival for up to 1 year. Statistical determinations were performed using t test analysis.
transformation, tumorigenesis, and metastasis in mice (12, 13, 30–32). Normal rat and human p185neu molecules have also been shown to be tumorigenic when expressed at very high density (11, 33–35); these molecules facilitate the formation of the p185neu dimer that is associated with increased tyrosine kinase activity, thereby contributing to cellular transformation (36). Transformation in vitro and tumorigenesis in vivo caused by either mutated activated HER-2/neu or overexpressed normal p185neu can be suppressed by anti-p185neu-antibody-mediated down-regulation of p185neu (37–42) or by transcription repression of HER-2/neu promoter (43–46). Furthermore, breast tumor development in transgenic mice that express neuT can be prevented by anti-p185neu antibody through decreasing tyrosine phosphorylation of p185neu (47). Our previous study showed that emodin can repress activated HER-2/neu-induced transformation phenotypes, including anchorage-dependent and -independent growth and metastasis-associated properties in vitro (48). In the present study, we showed that emodin can inhibit the growth of HER-2/neu-overexpressing MDA-MB-361 tumors in vivo (Figs. 3a and 4a) but does not produce the same effect on MDA-MB 231 cells, which express basal levels of p185neu (Figs. 3b and 4b). We also showed that emodin decreases tyrosine phosphorylation of HER-2/neu in these emodin-treated tumors (Figs. 6 and 7). Our results further demonstrated that p185neu-tyrosine kinase activity is critical for transformation and tumorigenesis; therefore, tyrosine kinase inhibitors such as emodin may be useful for treatment of HER-2/neu-overexpressing cancers.

Several early clinical studies suggest a relationship between HER-2/neu expression and chemoresistance. Data from three large clinical trials in breast cancer (49–53) suggest an association between HER-2/neu overexpression and resistance to chemotherapy. Their results indicated that node-negative breast cancer patients whose tumor contains HER-2/neu overexpression have a less favorable prognosis due to a lack of response to adjuvant cyclophosphamide, methotrexate, and 5-fluorouracil-based chemotherapy. Later, a study of HER-2/neu overexpression in epithelial ovarian cancer also demonstrated that patients whose tumors had the alteration were more likely to fail chemotherapy with cyclophosphamide and carboplatin (54). Recently, 76 patients with untreated metastatic breast carcinoma were entered in a multicenter Phase II clinical trial of the combination of Taxol and Adriamycin. The results of this study show that elevated circulating HER-2/neu in patients with metastatic breast cancer correlated with a poor response to chemotherapy (55). All these reports support the notion that HER-2/neu overexpression is associated with chemoresistance.

On the contrary, the reports from Klijn et al. (56) and Berns et al. (57) show that patients with metastatic breast cancer and amplification of the HER-2/neu gene had a superior response to CMF chemotherapy (75%) compared with patients without HER-2/neu amplification (45%), and the median length of progression-free survival from the start of chemotherapy was superior in patients whose tumors exhibited HER-2/neu amplification. Recently, Baselga et al. (58) reported that patients with advanced disease show association of HER-2/neu overexpression with poor prognosis, but the odds of HER-2/neu-positive patients responding clinically to taxanes were three times greater than those of HER-2/neu-negative patients. Furthermore, Gianni et al. (59) reported that patients with metastatic breast cancer and amplification of HER-2/neu appears to confer a higher probability of complete remission and possible longer duration of response to doxorubicin and taxanes. Thor et al. (60) also reported that a three-way interaction between HER-2/neu, p53, and dose of cyclophosphamide, doxorubicin, and fluorouracil (CAF), their study suggested that patients with both HER-2/neu and p53 alterations (~10%) derive the greatest benefit from dose-intensive CAF with 90% survival at 10 years. Therefore, the clinical data to date are somewhat controversial about the role of HER-2/neu overexpression in chemotherapy response.

The data from laboratory experimental studies are also controversial. Tsai et al. (61) demonstrated an association be-
tween HER-2/neu expression levels and intrinsic chemoresistance to six different chemotherapeutic drugs in different lung cancer cell lines; later, they further demonstrated that elevation of p185<sub>neu</sub> levels in HER-2/neu transfected lung cancer cells resulted in chemoresistance and a correlation between intrinsic chemoresistance and HER-2/neu gene expression, p53 gene mutations, and cell proliferation characteristics in lung cancer cells (62, 63). In HER-2/neu transfected-MCF7 breast cancer cells, no significant chemoresistance difference in response to either 5-fluorouracil or doxorubicin was seen, whereas HER-2/neu overexpression was associated with a 2–4-fold increase in resistance to cisplatin in estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER-2/neu (15). Recently, studies from Yu et al. (16) indicate that overexpression of HER-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms, and overexpression of both p185<sub>neu</sub> and p170 mdr-1 renders breast cancer cells highly resistant to Taxol (64). The results from Pegram et al. (65) show that HER-2/neu-overexpression is not sufficient to induce intrinsic pleomorphic drug resistance in vitro and in vivo, and changes in chemosensitivity profiles resulting from HER-2/neu transfection in breast and ovarian cancers observed in vitro were cell line specific.

The reason to cause these differences among these studies is not clear yet. Although further clinical investigation is required to clarify HER-2/neu overexpression-mediated chemoresistance, one possible interpretation as mentioned in the report by Muss et al. (66) is that HER-2/neu overexpression may confer resistance to chemotherapy, and escalation of the dose may overcome that resistance. Thus, patients whose tumors do not overexpress HER-2/neu may already be sensitive to the conventional dose of chemotherapy and will not gain benefit from high-dose chemotherapy. However, patients whose tumors overexpress HER-2/neu may benefit from treatment of higher dose chemotherapy because these tumors are resistant to conventional doses of chemotherapy. The results from different laboratory groups suggest that chemoresistance induced by HER-2/neu overexpression may be cell type and drug dependent; in human lung cancer cells, enhanced HER-2/neu expression results in resistance to DOX, cisplatin, and etoposide (23, 61, 62); in human breast cancer cells, overexpression of HER-2/neu is able to induce resistance to Taxol through blocking the Taxol-induced apoptosis (16, 64). The discrepancies in Yu et al. (64) and Pegram et al. (65) may be due to different methodologies or different levels of HER-2/neu in the cell lines used. The HER-2/neu level is higher in those cells of Yu’s group than

![Fig. 6](image-url) Immunohistochemical staining of representative tumor tissue sections taken from control and emodin-treated mice inoculated with MDA-MB-361 cells. Positive (red) staining indicates either expression of p185<sub>neu</sub> protein (upper panel: left, control; right, emodin treated) or levels of tyrosine phosphorylation (lower panel: left, control; right, emodin treated).
Pegram's group. It could be that the level of HER-2/neu overexpression needs to be above a certain threshold to confer significant chemoresistance. However, further systematic studies are required to clarify the question.

Under our experimental conditions, our previous results demonstrated that emodin inhibits tyrosine phosphorylation of human lung HER-2/neu transfectants, which are much more resistant to chemotherapeutic agents than the parental cell lines that express a normal amount of HER-2/neu and sensitizes these resistant cells to chemotherapeutic drugs in vitro (23). In the present study, we further demonstrated that the tyrosine kinase inhibitor emodin sensitizes HER-2/neu-overexpressing breast cancer cells to Taxol not only in vitro but also in vivo. Several reports also indicated that anti-p185^neo^ antibodies act synergistically with chemotherapeutic drugs to inhibit breast cancer and ovarian cancer cell growth in vitro (67–69) and in clinic (70) by down-regulation of p185^neo^.

Furthermore, Yen et al. (71) reported that anti-HER-2/neu antibody TAB 250 can enhance cisplatin cytotoxicity, and similar enhancement was observed when cells were exposed with tyrosine kinase inhibitors, herbimycin A and its analogue CP127374. The results from this study suggest that the HER-2/neu tyrosine kinase is required for induction of chemoresistance, and combined treatment of tyrosine kinase inhibitors and chemotherapeutic agents, such as Taxol, may provide an effective therapeutic strategy to kill HER-2/neu-overexpressing cancer cells, although the detailed mechanism of emodin sensitization of cells to Taxol in vivo remains to be elucidated.

REFERENCES

Fig. 7 Western blot analysis of levels of HER-2/neu tyrosine phosphorylation and expression of p185^neo^ protein in vivo. Protein extracts were prepared by homogenizing tumor tissues obtained from the control, emodin-treated, Taxol-treated, and combined emodin and Taxol-treated mice with lysis buffer. Western blotting was done using anti-phospho-emodin-treated, Taxol-treated, and combined emodin and Taxol-treated antibodies, as described in “Materials and Methods.” Right, MW, molecular weight (in thousands).


Tyrosine Kinase Inhibitor Emodin Suppresses Growth of HER-2/neu-overexpressing Breast Cancer Cells in Athymic Mice and Sensitizes These Cells to the Inhibitory Effect of Paclitaxel

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