

# Curcumin Inhibits Tyrosine Kinase Activity of p185<sup>neu</sup> and Also Depletes p185<sup>neu</sup><sup>1</sup>

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## ABSTRACT

Curcumin, a natural compound present in turmeric, possessing both anti-inflammatory and antioxidant effects, has been studied vigorously as a chemopreventative in several cancer models. The *erbB2/neu* gene-encoded p185<sup>neu</sup> tyrosine kinase is a potent oncoprotein. Overexpression of p185<sup>neu</sup> in breast cancer is known to be a poor prognostic factor. We investigated the effect of curcumin on p185<sup>neu</sup> tyrosine kinase and on the growth of breast cancer cell lines. Curcumin dose-dependently inhibited p185<sup>neu</sup> autophosphorylation and transphosphorylation *in vitro* and depleted p185<sup>neu</sup> protein *in vivo*. It dissociated the binding of p185<sup>neu</sup> with GRP94 (glucose-regulated protein), a molecular chaperone, and enhanced the depletion of p185<sup>neu</sup>. The amount of p185<sup>neu</sup> protein on the cell membrane was drastically decreased after curcumin treatment. These data demonstrated a new mechanism of the anti-tyrosine kinase activity of curcumin. The growth of several breast cancer cell lines was inhibited; the IC<sub>50</sub> ranged from 7 to 18  $\mu$ M, which, however, did not correlate with the expression level of p185<sup>neu</sup>. Colony formation in the soft agar assay, a hallmark of the transformation phenotype, was preferentially suppressed in p185<sup>neu</sup>-overexpressing cell lines by 5  $\mu$ M curcumin (% of control, basal level *versus* overexpression: 59.3 *versus* 16.7%;  $P < 0.001$  by Student's *t* test). Because curcumin effectively inhibited p185<sup>neu</sup> tyrosine kinase activity by depleting p185<sup>neu</sup> and potently suppressed the growth of multiple breast cancer cell lines, its therapeutic potential in advanced breast cancer is worthy of further investigation.

## INTRODUCTION

Curcumin is the major pigment in turmeric, a rhizome of the plant *Curcuma longa* Lin. It is used as a flavor (curry) and also as an herbal medicine for inflammatory disease. It displays anticarcinogenic properties in animals, as indicated by its ability to inhibit both tumor initiation induced by benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (1) and tumor promotion induced by phorbol esters (2), possibly by inhibiting protein kinase C (3). Curcumin also has anti-tyrosine kinase activity and has been shown to inhibit ligand-induced activation of the epidermal growth factor receptor tyrosine phosphorylation (4, 5) without affecting the protein level. The *erbB-2/neu* (also known as *HER-2*) gene encodes a 185-kDa protein that has tyrosine kinase activity (6). It is overexpressed in about 30% of breast cancers and is associated with poor prognosis (7). Transfecting breast epithelial cells with *erbB-2/neu* transforms these cells (8), and cancer cell lines transfected with *erbB-2/neu* show an enhanced ability to invade and metastasize (9–11). Animal model studies have shown that down-regulating p185<sup>neu</sup> by repressing the *erbB2/neu* promoter (12–14) or by using anti-p185<sup>neu</sup> antibodies (15–18) can suppress tumor growth and dissemination. An inhibitor of p185<sup>neu</sup> tyrosine kinase, emodin, also preferentially suppresses the growth of cell lines that overexpress p185<sup>neu</sup> (19). A clinical trial has shown that the humanized anti-p185<sup>neu</sup> monoclonal antibody also is effective (20). These findings suggest that manipulating p185<sup>neu</sup> may be of substantial value in the treatment of breast cancer. Because curcumin has already been shown to have low systemic toxicity in animal and human studies (21, 22), we explored the effect of curcumin on p185<sup>neu</sup> tyrosine kinase and found that it inhibited p185<sup>neu</sup> tyrosine kinase activity and, more importantly, depleted the protein itself. Curcumin dissociated the binding of p185<sup>neu</sup> with the GRP94<sup>3</sup> protein, a molecular chaperone in the endoplasmic reticulum (23) and accelerated depletion of mature p185<sup>neu</sup>. Curcumin also inhibited the growth of several breast cell lines and preferentially suppressed the colony formation of cell lines that overexpressed p185<sup>neu</sup>.

## MATERIALS AND METHODS

**Cell Culture.** The human breast cancer cell line AU-565 was obtained from the Naval Bioscience Laboratory (Oakland, CA). Other breast cancer cell lines—SKBr3, MDA-MB361, MDA-MB453, MDA-MB474, BT-483, MDA-MB231, MDA-MB435, MCF-7, and the immortalized breast cell line HBL—

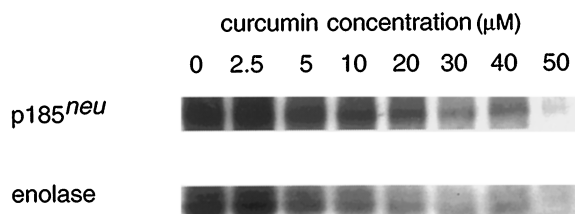
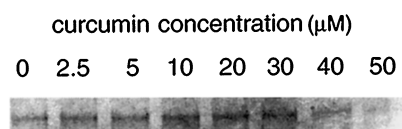
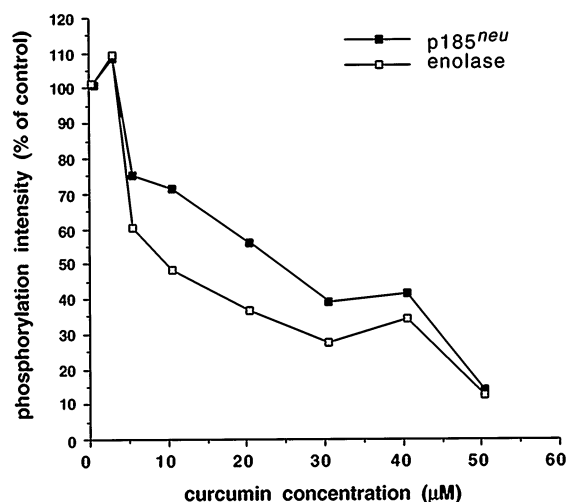
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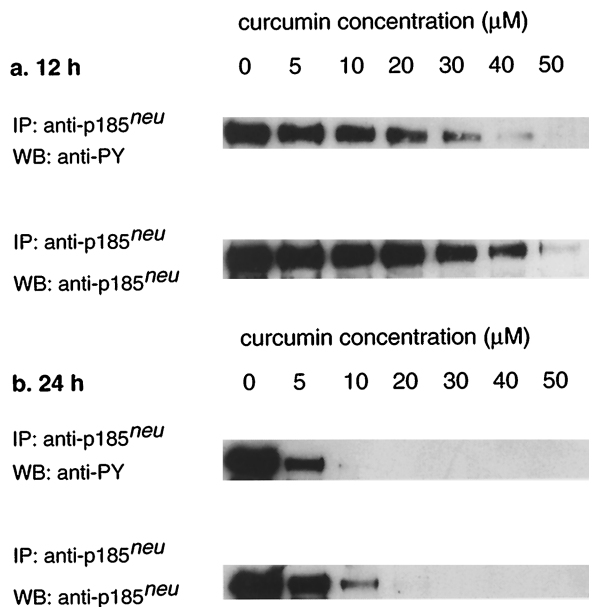
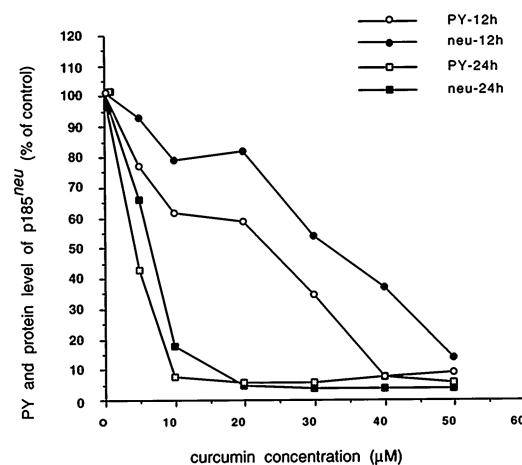
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<sup>3</sup> The abbreviations used are: GRP, glucose-regulated protein; RIPA buffer, 0.15 mM NaCl, 20 mM Na<sub>2</sub>PO<sub>4</sub> (pH 7.4), 1% Triton X-100, 0.1 M NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride; TESV buffer, 50 mM Tris (pH 7.5), 2 mM EDTA, 100 mM NaCl, and 1 mM vanadate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

**a. immunocomplex kinase assay****b. Coomassie blue stain****c.**

**Fig. 1** Inhibitory effect of curcumin on autophosphorylation and transphosphorylation of p185<sup>neu</sup> tyrosine kinase. In **a**, cell lysates from untreated AU565 cells were immunoprecipitated with anti-p185<sup>neu</sup> antibody. Kinase activity was measured by incubation with [ $\gamma$ -<sup>32</sup>P]ATP, enolase, and varying concentration of curcumin at room temperature. Reactants were resolved on 7.5% SDS-PAGE. The gel was dried, and the phosphorylation products were visualized by autoradiography. In **b**, Coomassie Brilliant Blue R250 staining of the same gel showed that approximately equal amounts of p185<sup>neu</sup> were immunoprecipitated. **c**, quantitative analysis of the results of autoradiography. The film was scanned and the bands were analyzed and quantified with NIH image software. The data were normalized against DMSO control.

100—were obtained from the American Type Culture Collection (Rockville, MD). SKBr3, MDA-MB361, MDA-MB453, MDA-MB474, BT-483, and AU-565 cells overexpress p185<sup>neu</sup>, whereas MDA-MB231, MDA-MB435, MCF-7, and HBL-100 cells express the basal level of p185<sup>neu</sup>. All of the cells were grown in DMEM/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and gentamicin (50 mg/ml). Cells were grown in a humidified incubator at 37°C under 5% CO<sub>2</sub> in air.

**c.**

**Fig. 2** Effect of dose dependence of curcumin treatment on the tyrosine phosphorylation and protein level of p185<sup>neu</sup> in AU565 cells. The cells were incubated without (0) or with (5 to 50  $\mu\text{M}$ ) curcumin at 37°C for 12 h (**a**) or 24 h (**b**). Cell extracts were immunoprecipitated by the anti-p185<sup>neu</sup> antibody and then Western blotted with either antiphosphotyrosine (anti-PY) antibody or anti-p185<sup>neu</sup> antibody. **c**, quantitative analysis of the results of Western blots. The film was scanned, and the bands were analyzed and quantified with NIH image software. The data were normalized against DMSO control.

**Immunoprecipitation and Western Blot Analysis.**

Cells were washed three times with PBS and then lysed in lysis buffer [20 mM Na<sub>2</sub> PO<sub>4</sub> (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1  $\mu\text{g/ml}$  aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{g/ml}$  leupeptin, 100 mM NaF, and 2 mM Na<sub>3</sub>VO<sub>4</sub>]. Protein content was determined against a standardized control, using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 500  $\mu\text{g}$  of protein was immunoprecipitated with a monoclonal anti-p185<sup>neu</sup> antibody [c-neu (Ab-3), Oncogene Science, Inc., Uniondale, NY] on protein A-Sepharose. The immune complexes were washed, separated by 7.5% SDS-

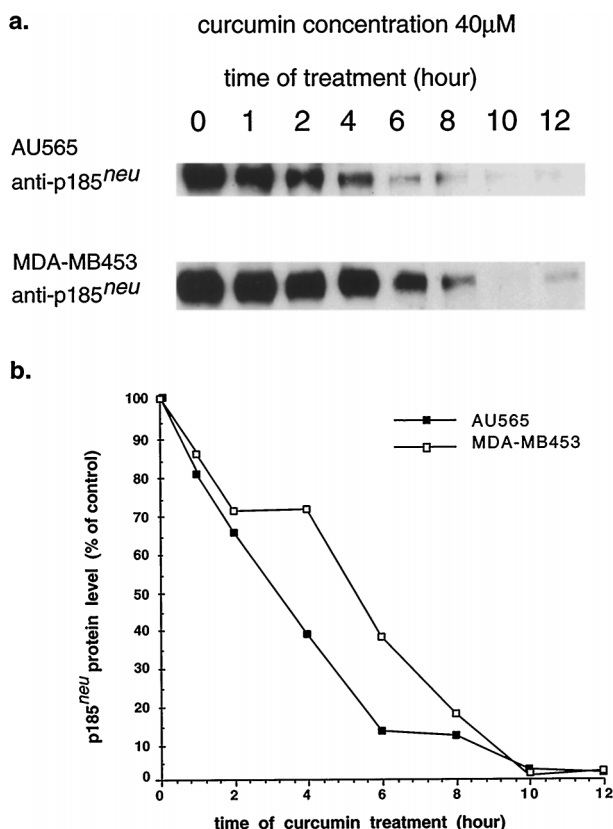


Fig. 3 a, time course of p185<sup>neu</sup> depletion in AU-565 cells and MDA-MB453 cells that were incubated with a 40-μM concentration of curcumin for varying time periods and then were harvested for Western blot analysis with anti-p185<sup>neu</sup>. b, quantitative analysis of the results of Western blots. The film was scanned, and the bands were analyzed and quantified with NIH image software. The data were normalized against control.

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-p185<sup>neu</sup> antibody, c-neu (Ab-3), to detect p185<sup>neu</sup> or antiphosphotyrosine antibody (UBI, Lake Placid, NY) to detect phosphotyrosine] and then incubated with horseradish peroxidase-conjugated goat antimouse antibody (1:2500 dilution; Boehringer Mannheim Corp., Indianapolis, IN); bands were visualized with the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). The intensity of the bands was scanned and quantified with NIH image software.

**Immunocomplex Kinase Assay.** The immunocomplex kinase assay was modified from those described previously (24). Immunocomplex—precipitated from lysate of AU565 cells with monoclonal anti-p185<sup>neu</sup> antibody c-neu (Ab-3) on protein-A-conjugated agarose (Boehringer Mannheim)—was washed three times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in assay buffer [50 mM Tris-HCl (pH 7.5) and 10 mM MnCl<sub>2</sub>]. To 40-μl beads (protein-A-conjugated agarose), 10

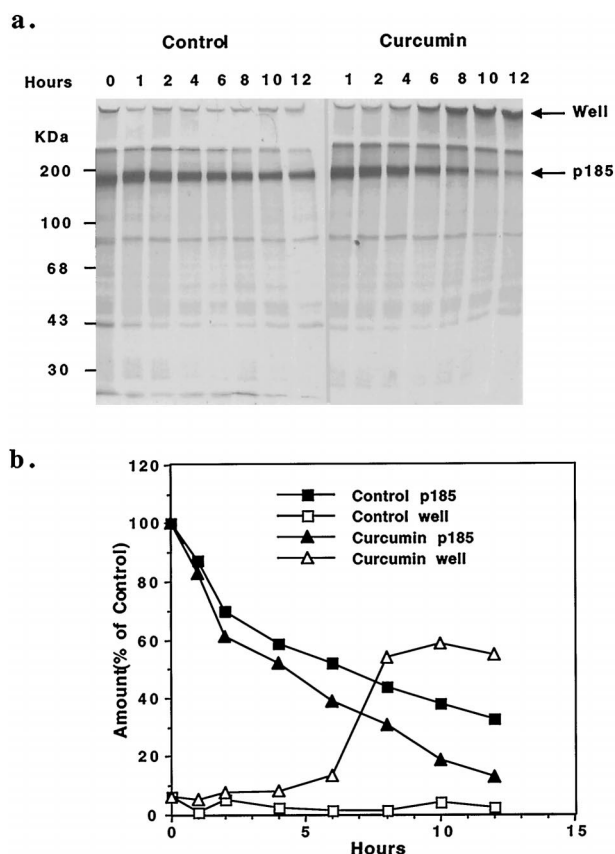
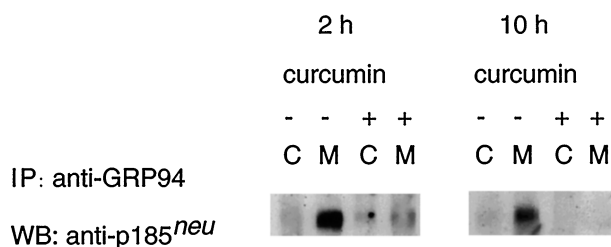


Fig. 4 AU-565 cells were pulsed with 0.1 mCi [<sup>35</sup>S]methionine for 30 min and chased in the absence or presence of 40 μM curcumin (a). Cells were lysed in RIPA buffer with 1% Triton X-100 at different times. Equal protein amounts were immunoprecipitated with anti-p185<sup>neu</sup> antibody and separated by 7.5% SDS-polyacrylamide gel. Fluorography was performed after the gel was dried. b, quantitative analysis of the results of autoradiography. The films were scanned, and the bands were analyzed and quantified with a Beckman DU-70 Spectrophotometer.

μCi [γ-<sup>32</sup>P]ATP (Amersham) and 10 μl of enolase with a concentration of 2.5 mg/ml (Sigma Chemical Co., St. Louis, MO) were added and incubated for 20 min at room temperature. The reactants were separated by 7.5% SDS-PAGE. The gel was dried and visualized by autoradiography.

**Pulse Labeling of AU-565 Cells.** AU-565 cells were grown to 70% confluence in 100-mm dishes in DMEM/F12 supplemented with 10% FCS. Plates were washed and then incubated in DMEM lacking methionine and cysteine for 20 min and then pulsed for 30 min in 1 ml of deficient media containing 10% dialyzed FCS and 0.1 mCi [<sup>35</sup>S]methionine (Trans-Label, ICN). After pulsing, plates were washed once in complete media and then incubated in complete media containing either 40 μM curcumin or vehicle control (0.1% DMSO). After incubation, plates were washed three times in PBS, and then the cells were lysed in RIPA buffer. Cell lysates were cleared by a 10-min spin at 12,000 × g, and then an equal amount of protein (500 μg) was immunoprecipitated with monoclonal antibody Ab3 as described above. Immunocomplexes were solubilized with SDS sample buffer and run on a 7.5% Laemmli gel. After treating the



**Fig. 5** Dissociation of p185<sup>neu</sup> from GRP94 and the induction of depletion of p185<sup>neu</sup>. AU565 cells were incubated 2 h or 10 h in the presence of 40  $\mu$ M curcumin or control vehicle (DMSO). The proteins contained in membrane (M) or cytosol (C) portions were subjected to immunoprecipitation with rat monoclonal antihuman GRP 94. The proteins then were separated by SDS-PAGE and immunoblotted with monoclonal antibody to p185<sup>neu</sup>.

gel with Enhance(New England Nuclear), the gel was dried and subjected to fluorography. X-ray films were scanned and quantified on a Beckman DU-70 spectrophotometer.

**Preparation of Cytosol and Crude-Membrane Fractions from AU-565 Cells.** AU-565 cells ( $10^7$ ) were plated in 150-mm plastic dishes. After treatment, the cells were washed twice with ice-cold PBS and scraped with a cell scraper into 1 ml of ice-cold TESV buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1  $\mu$ g/ml aprotinin). Cells were homogenized using a Dounce tissue homogenizer and then lysed by sonication on ice three times for 10 s each; the samples were chilled in ice water between sonications. The cell lysate was ultracentrifuged at high speed ( $100,000 \times g$ , 60 min,  $4^\circ\text{C}$ ) for preparation of membrane and cytosol fractions. The supernatant fraction from the high-speed centrifugation (cytosol) was saved, and the crude membrane pellet was resuspended by sonication in 0.5 ml ice-cold TESV buffer containing protease inhibitors. The membrane suspension was then clarified by centrifugation at  $12,000 \times g$  for 15 min ( $4^\circ\text{C}$ ), and the supernatant (soluble membrane fraction) was saved.

**Coimmunoprecipitation of GRP94 and p185<sup>neu</sup> and Western Immunoblot.** GRP94 protein was immunoprecipitated from both membrane and cytosol fractions (500  $\mu$ g of protein, prepared fresh) using 2  $\mu$ g of rat monoclonal antihuman GRP94 antibody (StressGen, Victoria B.C., Canada) with an incubation period of 1 h at  $4^\circ\text{C}$ . Rabbit antirat protein A-Sepharose beads, preswollen and equilibrated in TESV, were added, and samples were rotated in a tumbler for 40 min at  $4^\circ\text{C}$ . The beads were washed three times with TESV buffer, resuspended in  $1 \times$  sample loading buffer [0.0627 M Tris-HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.0005% bromphenol blue], and heated for 5 min at  $100^\circ\text{C}$ . The immunoprecipitate was then subjected to Western blot analysis with a monoclonal anti-p185<sup>neu</sup> antibody [c-neu (Ab-3), Oncogene Science].

**Immunofluorescence Study of Subcellular Localization of p185<sup>neu</sup> in the Presence or Absence of Curcumin.** AU565 cells were grown on coverslips, rinsed with PBS, fixed and permeabilized with methanol at room temperature for 5 min, and rinsed again with PBS. After being blocked with 3% albumin, the coverslips were overlaid with p185<sup>neu</sup> antibody (Ab-3, 5  $\mu$ g/ml in PBS, Oncogene Science) and kept at  $37^\circ\text{C}$  for 1 h.

After a wash in PBS, the coverslips were overlaid with rhodamine-conjugated goat antimouse immunoglobulin (1:1000 in PBS; Pierce) and kept at  $37^\circ\text{C}$  for an additional hour. After being rinsed in PBS and water, the coverslips were air-dried and mounted. Fluorescence was visualized using a Nikon Optiphot-2 microscope.

**Proliferation Assay.** Cells were detached by trypsinization, seeded at  $2 \times 10^4$  cells/ml in a 96-well microtiter plate overnight, then treated with varying concentrations of curcumin, and incubated for an additional 72 h. The effect of curcumin on cell growth was examined by the MTT growth assay (25, 26). Briefly, 20  $\mu$ l of MTT solution (5 mg/ml; Sigma) was added to each well and incubated for 4 h at  $37^\circ\text{C}$ . The supernatant was aspirated, and the MTT formazan that was formed by metabolically viable cells was dissolved in 150  $\mu$ l of DMSO, then monitored by a microplate reader (Dynatech MR 5000 fluorescence, Dynatech Corp., Burlington, MA) at a wavelength of 590 nm.

**Colony Formation in Soft Agarose.** Cells ( $1 \times 10^3$  cells/well) were seeded in 24-well plates in culture medium containing 0.35% low-melting agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer in the presence of varying concentration of curcumin or control vehicle and incubated for 3 weeks at  $37^\circ\text{C}$ . Colonies were then stained with *p*-iodonitrotetrazolium violet (1 mg/ml), and colonies larger than 100  $\mu$ m were counted. Each determination was performed three times. The difference in effects of curcumin between groups expressing baseline levels and those that overexpressed p185<sup>neu</sup> was evaluated using Student's *t* test.

## RESULTS

### Curcumin-inhibited p185<sup>neu</sup> Tyrosine Kinase *in Vitro*.

To examine the effect of curcumin on the tyrosine kinase activity of p185<sup>neu</sup>, an immunocomplex kinase assay was performed. Immunoprecipitates of lysate of AU565 cells were incubated in the presence of varying concentrations of curcumin or control vehicle. Both the autophosphorylation of p185<sup>neu</sup> and the transphosphorylation of an exogenous substrate, enolase, were inhibited by curcumin in a dose-dependent manner (Fig. 1a). As a control, staining the same gel with Coomassie Brilliant Blue R250 showed that the amounts of p185<sup>neu</sup> immunoprecipitated were approximately equal (Fig. 1b). These results demonstrated that curcumin repressed the intrinsic tyrosine kinase activity of p185<sup>neu</sup>. The *in vitro* IC<sub>50</sub> for autophosphorylation and transphosphorylation was 24 and 9  $\mu$ M, respectively (Fig. 1c).

### *In Vivo* Effect of Curcumin on p185<sup>neu</sup> Phosphorylation and Protein Level.

To test the effect of curcumin on living cells, AU-565 cells were treated with varying concentrations of curcumin or control vehicle for 12 or 24 h. The lysate was then immunoprecipitated by anti-p185<sup>neu</sup> antibody and subsequently blotted with antiphosphotyrosine antibody to detect the level of tyrosine phosphorylation or with anti-p185<sup>neu</sup> antibody to monitor the p185<sup>neu</sup> protein level. Curcumin inhibited tyrosine phosphorylation and, unexpectedly, depleted p185<sup>neu</sup> in a concentration-dependent manner (Fig. 2, a and b). After quantitation and normalization with control, it became evident that the decrease in p185<sup>neu</sup> protein itself could account for most of the decrease



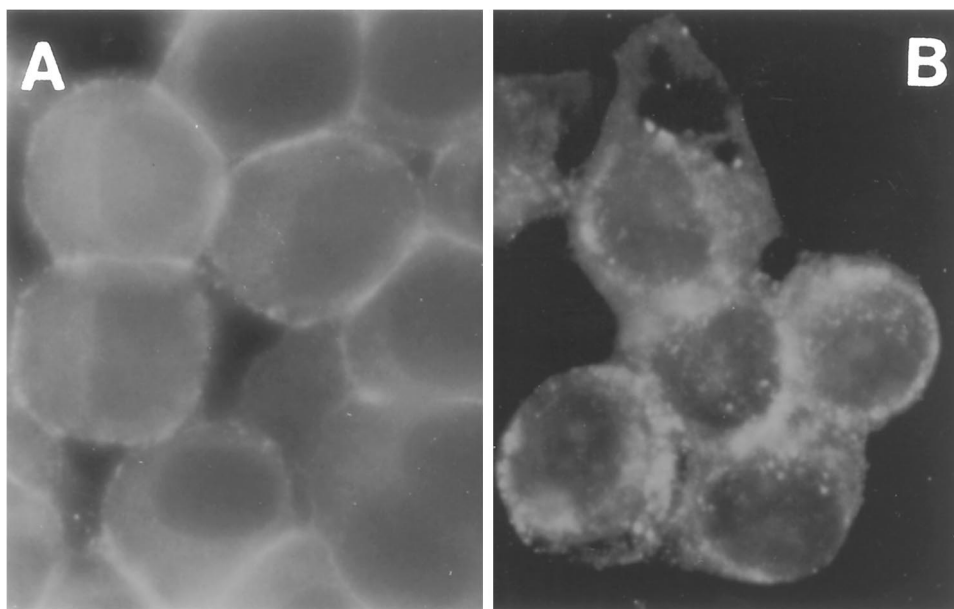


Fig. 6 Changes in subcellular distribution of p185<sup>neu</sup> after the 10-h exposure to curcumin. Cells grown on coverslips were treated with curcumin 40  $\mu$ M (A) or control vehicle (B) for 10 h. After fixation and permeabilization with methanol, an anti-p185<sup>neu</sup> immunofluorescence study was performed with a rhodamine-conjugated secondary antibody. Fluorescence was visualized using a Nikon Optiphot-2 microscope.

in the phosphorylation of p185<sup>neu</sup>, especially after a 24-h treatment (Fig. 2c). *In vivo*, therefore, the depletion of p185<sup>neu</sup> itself is dominant over the inhibition of p185<sup>neu</sup> kinase activity. After the 12-h treatment, the IC<sub>50</sub> of inhibiting p185<sup>neu</sup> phosphorylation and depleting p185<sup>neu</sup> protein was approximately 23  $\mu$ M and 32  $\mu$ M, respectively. The IC<sub>50</sub> for the 24-h treatment was 4 and 6.5  $\mu$ M, respectively. Western blots of lysate also showed similar results (data not shown).

#### Kinetics of p185<sup>neu</sup> Depletion in AU-565 by Curcumin.

To investigate the kinetics of depletion of p185<sup>neu</sup>, we treated AU-565 cell lines with 40  $\mu$ M curcumin for different time periods and then harvested them for Western blot analysis of p185<sup>neu</sup>. The p185<sup>neu</sup> protein level began to decrease after curcumin treatment and reached nadir after 8–10 h, at which time, p185<sup>neu</sup> could almost not be detected (Fig. 3a). We also demonstrated similar results in another breast cancer cell line overexpressing p185<sup>neu</sup>, MDA-MB453 (Fig. 3a). Less than one-half of the p185<sup>neu</sup> remained after 4–6 h of curcumin treatment (Fig. 3b).

#### Curcumin Enhanced Depletion of Mature p185<sup>neu</sup> and Led to Accumulation of a High Molecular Weight Product.

The pulse-chase study by labeling with [<sup>35</sup>S]methionine for 30 min clearly showed that 40  $\mu$ M curcumin did enhance the depletion of mature p185<sup>neu</sup> (Fig. 4a). Interestingly, an increasing amount of signal was observed in the loading wells as time passed. After 8 h of treatment with curcumin, the intensity of signal in the loading well actually was higher than that of mature p185<sup>neu</sup> (Fig. 4b). Because it increased gradually while the mature p185<sup>neu</sup> decreased, the signal may have been coming from modified p185<sup>neu</sup> rather than from another associated protein.

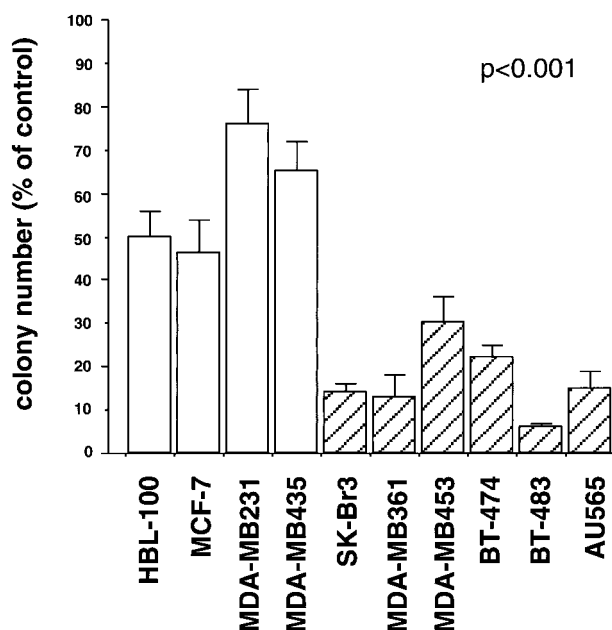
**Dissociation of p185<sup>neu</sup> from GRP94 Preceded the Depletion of p185<sup>neu</sup>.** To further study the mechanism of p185<sup>neu</sup> depletion, we treated the AU-565 cells with either the control vehicle (DMSO) or 40  $\mu$ M curcumin. After 2 or 10 h, we

harvested them for fractionation into cytosol and membrane portions and studied the binding of p185<sup>neu</sup> with GRP94, a molecular chaperone, because binding with GRP94 helps to stabilize p185<sup>neu</sup> in the endoplasmic reticulum during transportation (27). Equal amounts of fractionated proteins were immunoprecipitated with 2  $\mu$ g of anti-GRP94 monoclonal antibody, and the immunoprecipitates were then blotted with anti-p185<sup>neu</sup> antibody to detect p185<sup>neu</sup>. After the 2-h treatment, when the protein level had not significantly changed (data not shown, and Fig. 3 and Fig. 4), the binding of p185<sup>neu</sup> with GRP94 had already markedly decreased (Fig. 5). By the 10-h treatment, no detectable p185<sup>neu</sup> was found to be associated with GRP94 (Fig. 5). The protein level of GRP94 showed no change after curcumin treatment (data not shown). This result suggested that curcumin dissociated the binding of p185<sup>neu</sup> with GRP94 first, and this may consequently have led to the depletion of p185<sup>neu</sup>.

#### Curcumin Increased the Intensity of Immunofluorescence of Anti-p185<sup>neu</sup> and Changed the Subcellular Distribution of p185<sup>neu</sup>.

An immunofluorescent study with anti-p185<sup>neu</sup>, Ab-3, showed that the control cells had strong immunofluorescence at the plasma membrane (Fig. 6A). After curcumin treatment, the immunofluorescence at the plasma membrane disappeared and was replaced by diffuse cytoplasmic punctate staining (Fig. 6B), which might be compatible with localization in the endoplasmic reticulum or the Golgi apparatus. There was no change in the amount or distribution of GRP94 after curcumin treatment (data not shown). The addition of cycloheximide did not significantly alter the effect of curcumin on the immunostaining pattern (data not shown), which indicated that *de novo* synthesis of p185<sup>neu</sup> was not required for the cytoplasmic punctate staining.

**Effect of Curcumin on the Proliferation of Human Breast Cancer Cells.** A panel of breast cell lines was used to test the effect of curcumin on cell growth. SKBr3, MDA-MB361, MDA-MB453, MDA-MB474, BT-483, and AU-565



**Fig. 7** Effect of curcumin on anchorage-independent growth of breast cancer cells. Cells ( $1 \times 10^3$  cells/well) were seeded in 24-well plates in culture medium containing 0.35% low-melting agarose over a 0.7% agarose layer in the presence of 5 mM of curcumin or control vehicle and were incubated for 3 weeks at 37°C. Colonies were then stained with *p*-iodonitrotetrazolium violet (1 mg/ml), and colonies larger than 100 mm were counted. The percentage of colony formation was calculated by defining the number of colonies in the absence of curcumin as 100%. Each determination was performed three times. The difference in effects of curcumin between groups with baseline (□) and overexpression (▨) levels of p185<sup>neu</sup> was evaluated with Student's *t* test.

are p185<sup>neu</sup>-overexpressing breast cancer cell lines. MCF-7, MDA-MB231, and MDA-MB435 are three human breast cancer cell lines expressing the basal level of p185<sup>neu</sup>. The HBL-100 cell line is derived from normal human breast tissue transformed by SV40 large T antigen and expresses a basal level of p185<sup>neu</sup>. The MTT growth assay showed that curcumin is a growth inhibitor to multiple breast cancer cell lines, regardless of their expression level of p185<sup>neu</sup>, which suggests that curcumin may be able to suppress cell proliferation through mechanisms independent of p185<sup>neu</sup> overexpression. The range of IC<sub>50</sub> is from 5 to 18 μM. MCF-7 and MDA-MB453 are the most resistant lines with an IC<sub>50</sub> of 18 μM (data not shown).

**Effect of Curcumin on Anchorage-independent Growth of Breast Cancer Cells.** To determine whether curcumin may affect anchorage-independent growth, an important hallmark of the transformation phenotype, we seeded cells into soft agarose in the presence of control vehicle or varying concentrations of curcumin and monitored them for colony formation. At a 5-μM concentration of curcumin, the colony-forming activity of p185<sup>neu</sup>-overexpressing breast cancer cells was significantly more suppressed than that of nonoverexpressing cell lines (% of control, basal level *versus* overexpressing: 59.3 *versus* 16.7%;  $P < 0.001$  by Student's *t* test; Fig. 7). At 10 μM curcumin and more, colony formation was almost completely suppressed in all of the cell lines tested. This finding implies that at low concen-

trations of curcumin, p185<sup>neu</sup> is likely to be the primary target for the reduction of colony formation. At higher concentrations of curcumin, other mechanisms may apply, such as cell cycle regulation and/or an induction of apoptosis. The results also suggest that p185<sup>neu</sup> is important in the transformation phenotype of p185<sup>neu</sup>-overexpressing cell lines.

## DISCUSSION

In this study, we demonstrated that curcumin inhibits p185<sup>neu</sup> tyrosine kinase activity *in vitro* and depletes mature p185<sup>neu</sup> *in vivo*. After 10 h of curcumin treatment, the p185<sup>neu</sup> at the plasma membrane was almost undetectable. Curcumin dissociates the complex between p185<sup>neu</sup> and GRP94, a molecular chaperone, in the endoplasmic reticulum. GRP94 is known to associate with, and to stabilize, p185<sup>neu</sup> (27), and this dissociation precedes the depletion of mature p185<sup>neu</sup> at the plasma membrane. The depletion of mature membrane p185<sup>neu</sup> and the concomitant accumulation of p185<sup>neu</sup> in the cytoplasmic organelles is compatible with the notion that the complex of p185<sup>neu</sup> with GRP94 is necessary for its maturation and subsequent transport to the plasma membrane (27). However, the blockade of recruitment of p185<sup>neu</sup> to the plasma membrane cannot entirely explain the effect of curcumin on the mature p185<sup>neu</sup>, inasmuch as the half-life of p185<sup>neu</sup> is estimated to be 7 h (28). Geldanamycin was shown to deplete mature p185<sup>neu</sup> by the mediation of polyubiquitination and proteasomal degradation, which occur within minutes of exposure (29). In Fig. 4*b*, the accumulation of signal in the loading well as mature p185<sup>neu</sup> decreased suggested that it might come from modified protein with very large molecular weight. Ubiquitination may be implicated with this phenomenon as well as other unknown mechanisms. Further study is required to examine this phenomenon and to systematically test this possibility.

This is the first report showing that curcumin can inhibit protein kinase activity by depleting the protein kinase itself. Among the presently available tyrosine kinase inhibitors, curcumin is the only natural compound known to inhibit tyrosine kinase activity and also to deplete the tyrosine kinase protein itself. Natural inhibitors such as emodin (19) and genistein (28), or synthetic tyrphostins (30), can inhibit p185<sup>neu</sup> tyrosine kinase activity, but the protein level is not changed. Ansamycins are known to deplete several tyrosine kinases but do not inhibit kinase activity directly (27, 31). On the basis of the facts that virtually all of the natural phosphotyrosine-receptor kinase blockers are ATP competitors (32) and that curcumin can inhibit multiple protein kinases (3, 5), it is possible that curcumin may also act as an ATP competitor. GRP94 is also an ATP-binding protein and has Mg<sup>2+</sup>-dependent ATPase activity (33). Curcumin may disrupt the association of GRP94 with p185<sup>neu</sup> through competition with ATP, and this may explain why curcumin can both inhibit kinase activity and deplete p185<sup>neu</sup>. Curcumin also can deplete other proteins, such as erbB-3 and erbB-4, perhaps through a similar mechanism (data not shown).

The effect of curcumin on the growth inhibition of breast cell lines was very similar except for the MCF-7 and MDA-MB453, which were relatively resistant. The range of IC<sub>50</sub> of growth of breast cancer cell lines was close to that of those depleting p185<sup>neu</sup> protein or inhibiting p185<sup>neu</sup> kinase but was

not correlated with expression of p185<sup>neu</sup>. This suggested that the effect of growth inhibition was not dependent on the blockade of the p185<sup>neu</sup> signal-transduction pathway. As for the effect of curcumin on the transformation phenotype, at 5  $\mu$ M, the colony formation of p185<sup>neu</sup>-overexpressing cell lines was significantly more suppressed than that of the nonoverexpressing cell lines in soft agar. At higher concentrations (data not shown), the colony-forming ability was almost completely suppressed, regardless of the level of p185<sup>neu</sup> expression. The results indicated that curcumin preferentially suppressed the transformation ability of p185<sup>neu</sup>-overexpressing cancer cells at lower concentrations. However, other effects of curcumin, including the inhibition of other kinases and the depletion of other proteins, may account for its inhibitory effect on anchorage-independent growth of cell lines expressing the basal level of p185<sup>neu</sup>.

Some effects of curcumin on cell cycle regulation and the induction of apoptosis have been published. Curcumin is known to prevent the activation of NF- $\kappa$ B and down-regulate AP-1 binding factors (34), possibly by interfering with signaling molecules in the JNK pathway (35). Low concentration of curcumin is known to arrest cell proliferation in the G<sub>0</sub>-G<sub>1</sub> phase, whereas high concentration of curcumin induces apoptosis in rat A7r5 cells (36). A curcumin-induced apoptosis in human basal cells was shown to be dependent on a p53 signaling pathway (37). Curcumin was shown to accumulate in plasma membrane, endoplasmic reticulum, and nuclear envelope and to produce apoptosis-like changes in plasma membranes in rat thymocytes (38).

In this study, we have demonstrated that curcumin inhibited p185<sup>neu</sup> *in vitro* and depleted p185<sup>neu</sup> protein *in vivo* by disrupting its binding with a chaperone, GRP94. This is a newly discovered mechanism of the anti-tyrosine kinase effect of curcumin. We have also shown the inhibitory effect of curcumin on the growth and colony formation of several breast cancer cell lines. The IC<sub>50</sub> of curcumin on several breast cancer cell lines is close to that of some chemotherapeutic agents such as 5-fluorouracil.<sup>4</sup> Animal and human studies have shown that the systemic toxicity of curcumin is minimal (21, 22). Therefore, in addition to its activity as a chemopreventive agent, the potential therapeutic role of curcumin for advanced cancers is worthy of further study.

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## REFERENCES

- Huang, M. T., Wang, Z. Y., Georgiadis, C. A., Laskin, J. D., and Conney, A. H. Inhibitory effects of curcumin on tumor initiation by benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene. *Carcinogenesis (Lond.)*, **13**: 2183–2186, 1992.
- Huang, M. T., Ma, W., Lu, Y. P., Chang, R. L., Fisher, C., Manchand, P. S., Newmark, H. L., and Conney, A. H. Effects of

curcumin, demethoxycurcumin, bisdemethoxycurcumin and tetrahydrocurcumin on 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion. *Carcinogenesis (Lond.)*, **16**: 2493–2497, 1995.

- Liu, J. Y., Lin, S. J., and Lin, J. K. Inhibitory effects of curcumin on protein kinase C activity induced by 12-*O*-tetradecanoyl-phorbol-13-acetate in NIH 3T3 cells. *Carcinogenesis (Lond.)*, **14**: 857–861, 1993.
- Korutla, L., and Kumar, R. Inhibitory effect of curcumin on epidermal growth factor receptor kinase activity in A431 cells. *Biochim. Biophys. Acta*, **1224**: 597–600, 1994.
- Korutla, L., Cheung, J. Y., Mendelsohn, J., and Kumar, R. Inhibition of ligand-induced activation of epidermal growth factor receptor tyrosine phosphorylation by curcumin. *Carcinogenesis (Lond.)*, **16**: 1741–1745, 1995.
- Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. Similarity of protein encoded by the human *c-erb-B-2* gene to epidermal growth factor receptor. *Nature (Lond.)*, **319**: 230–234, 1986.
- Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science (Washington DC)*, **235**: 177–182, 1987.
- Chazin, V. R., Kaleko, M., Miller, A. D., and Slamon, D. J. Transformation mediated by the human *HER-2* gene independent of the epidermal growth factor receptor. *Oncogene*, **7**: 1859–1866, 1992.
- Yu, D. H., and Hung, M. C. Expression of activated rat *neu* oncogene is sufficient to induce experimental metastasis in 3T3 cells. *Oncogene*, **6**: 1991–1996, 1991.
- Yu, D., Wolf, J. K., Scanlon, M., Price, J. E., and Hung, M. C. Enhanced *c-erbB-2/neu* expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res.*, **53**: 891–898, 1993.
- Yu, D., Wang, S.-S., Dulski, K. M., Tsai, C.-M., Nicolson, G. L., and Hung, M. C. *c-erbB-2/neu* overexpression enhances metastatic potential of human lung cancer cells by induction of metastasis-associated properties. *Cancer Res.*, **54**: 3260–3266, 1994.
- Xing, X., Matin, A., Yu, D., Xia, W., Sorgi, F., Huang, L., and Hung, M. C. Mutant SV40 large T antigen as a therapeutic agent for *HER-2/neu* overexpressing ovarian cancer. *Cancer Gene Ther.*, **3**: 168–174, 1996.
- Yu, D., Matin, A., Xia, W., Sorgi, F., Huang, L., and Hung, M. C. Liposome-mediated *in vivo* E1A gene transfer suppressed dissemination of ovarian cancer cells that overexpress *HER-2/neu*. *Oncogene*, **11**: 1383–1388, 1995.
- Zhang, Y., Yu, D., Xia, W., and Hung, M. C. *HER-2/neu*-targeting cancer therapy via adenovirus-mediated E1A delivery in an animal model. *Oncogene*, **10**: 1947–1954, 1995.
- Drebin, J. A., Link, V. C., Weinberg, R. A., and Greene, M. I. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. *Proc. Natl. Acad. Sci. USA*, **83**: 9129–9133, 1986.
- Drebin, J. A., Link, V. C., and Greene, M. I. Monoclonal antibodies reactive with distinct domains of the *neu* oncogene-encoded p185 molecule exert synergistic anti-tumor effect *in vivo*. *Oncogene*, **2**: 273–277, 1988.
- Katsumata, M., Okudaira, T., Samanta, A., Clark, D. P., Drebin, J. A., Jolicoeur, P., and Greene, M. I. Prevention of breast tumour development *in vivo* by downregulation of the p185<sup>neu</sup> receptor. *Nat. Med.*, **1**: 644–648, 1995.
- Park, J. W., Hong, K., Carter, P., Asgari, H., Guo, L. Y., Keller, G. A., Wirth, C., Shalaby, R., Kotts, C., Wood, W. I., Papahadjopoulos, D., and Benz, C. C. Development of anti-p185HER2 immunoliposomes for cancer therapy. *Proc. Natl. Acad. Sci. USA*, **92**: 1327–1331, 1995.
- Zhang, L., Chang, C.-j., Bacus, S. S., and Hung, M. C. Suppressed transformation and induced differentiation of *HER-2/neu*-overexpressing breast cancer cells by emodin. *Cancer Res.*, **55**: 3890–3896, 1995.
- Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore,

<sup>4</sup> Unpublished observations.

- J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with *HER2/neu*-overexpressing metastatic breast cancer. *J. Clin. Oncol.*, 14: 737–744, 1996.
21. Rao, C. V., Rivenson, A., Simi, B., and Reddy, B. S. Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res.*, 55: 259–266, 1995.
22. Soni, K. B., and Kuttan, R. Effect of oral curcumin administration on serum peroxides and cholesterol levels in human volunteers. *Indian J. Physiol. Pharmacol.*, 36: 273–275, 1992.
23. Mazzarella, R. A., and Green, M. ERp99, an abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90-kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (GRP94). *J. Biol. Chem.*, 262: 8875–8883, 1987.
24. Kiyokawa, N., Yan, D. H., Brown, M. E., and Hung, M. C. Cell cycle-dependent regulation of p185<sup>neu</sup>: a relationship between disruption of this regulation and transformation. *Proc. Natl. Acad. Sci. USA*, 92: 1092–1096, 1995.
25. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65: 55–63, 1983.
26. Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A., and Boyd, M. R. Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J. Natl. Cancer Inst.*, 82: 1113–1118, 1990.
27. Chavany, C., Mimnaugh, E., Miller, P., Bitton, R., Nguyen, P., Trepel, J., Whitesell, L., Schnur, R., Moyer, J., and Neckers, L. p185<sup>erbB2</sup> binds to GRP94 *in vivo*: dissociation of the p185<sup>erbB2</sup>/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185<sup>erbB2</sup>. *J. Biol. Chem.*, 271: 4974–4977, 1996.
28. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, 262: 5592–5595, 1987.
29. Mimnaugh, E., Chavany, C., and Neckers, L. Polyubiquitination and proteasomal degradation of the p185<sup>c-erbB-2</sup> receptor protein-kinase induced by geldanamycin. *J. Biol. Chem.*, 271: 22796–22801, 1996.
30. Levitzki, A. Tyrosine kinases: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB J.*, 3275–3282, 1992.
31. Miller, P., Schnur, R. C., Barbacci, E., Moyer, M. P., and Moyer, J. D. Binding of benzoquinoid ansamycins to p100 correlates with their ability to deplete the *erbB2* gene product p185. *Biochem. Biophys. Res. Commun.*, 201: 1313–1319, 1994.
32. Levitzki, A., and Gazit, A. Tyrosine kinase inhibition: an approach to drug development. *Science (Washington DC)*, 267: 1782–1788, 1995.
33. Li, Z., and Srivastava, P. K. Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation. *EMBO J.*, 12: 3143–3151, 1993.
34. Singh, S., and Aggarwal, B. B. Activation of transcription factor NF- $\kappa$ B is suppressed by curcumin (diferuloylmethane). *J. Biol. Chem.*, 270: 24995–25000, 1995.
35. Chen, Y. R., and Tan, T. H., Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene*, 17: 173–178, 1998.
36. Chen, H. W., and Huang, H. C., Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. *Br. J. Pharmacol.*, 124: 1029–1040, 1998.
37. Jee, S. H., Shen, S. C., Tseng, C. R., Chiu, H. C., and Kuo, M. L. Curcumin induces a p53-dependent apoptosis in human basal cell carcinoma cells. *J. Invest. Dermatol.*, 111: 656–661, 1998.
38. Jaruga, E., Salvioli, S., Dobrucki, J., Chrul, S., Bendorowicz-Pikula, K., Siskora, E., Franceschi, C., Cossarizza, A., and Bartosz, G., Apoptosis-like, reversible changes in plasma membrane asymmetry and permeability, and transient modifications in mitochondrial membrane potential induced by curcumin in rat thymocytes. *FEBS Lett.*, 433: 287–293, 1998.