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Nordihydroguaiaretic Acid (NDGA), an Inhibitor of the HER2 and IGF-1 Receptor Tyrosine Kinases, Blocks the Growth of HER2-Overexpressing Human Breast Cancer Cells

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Abstract We have reported that nordihydroguaiaretic acid (NDGA) inhibits the tyrosine kinase activities of the IGF-1 receptor (IGF-1R) and the HER2 receptor in breast cancer cells. Herein, we studied the effects of NDGA on the growth of estrogen receptor (ER) positive MCF-7 cells engineered to overexpress HER2 (MCF-7/HER2-18). These cells are an in vitro model of HER2-driven, ER positive, tamoxifen resistant breast cancer. NDGA was equally effective at inhibiting the growth of both parental MCF-7 and MCF-7/HER2-18 cells. Half maximal effects for both cell lines were in the 10–15 μM range. The growth inhibitory effects of NDGA were associated with an S phase arrest in the cell cycle and the induction of apoptosis. NDGA inhibited both IGF-1R and HER2 kinase activities in these breast cancer cells. In contrast, Gefitinib, an epidermal growth factor receptor inhibitor but not an IGF-1R inhibitor, was more effective in MCF-7/HER2-18 cells than in the parental MCF-7 cells and IGF binding protein-3 (IGFBP-3) was more effective against MCF-7 cells compared to MCF-7/HER2-18. MCF-7/HER2-18 cells are known to be resistant to the effects of the estrogen receptor inhibitor, tamoxifen. Interestingly, NDGA not only inhibited the growth of MCF-7/HER2-18 on its own, but it also demonstrated additive growth inhibitory effects when combined with tamoxifen. These studies suggest that NDGA may have therapeutic benefits in HER2-positive, tamoxifen resistant, breast cancers in humans. J. Cell. Biochem. 103: 624–635, 2008.

Key words: NDGA; receptor tyrosine kinase; HER2; IGF-1R; breast cancer

HER2 is a member of the EGFR family of type I receptor tyrosine kinases (RTK) [Yarden and Sliwkowski, 2001; Gschwind et al., 2004]. Overexpression of HER2 occurs in 25–30% of breast cancers and is associated with an aggressive tumor phenotype [Slamon et al., 1987, 1989]. Several studies have shown that upregulation of HER2 confers resistance to anti-estrogens

Abbreviations used: NDGA, nordihydroguaiaretic acid; IGF, insulin-like growth factor; IGFBP-3, insulin like growth factor binding protein 3; RTK, receptor tyrosine kinase.

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such as tamoxifen [Dowsett, 2001; Kurokawa and Arteaga, 2003; Shou et al., 2004; Gutierrez et al., 2005; Osborne et al., 2005] as well as several chemotherapeutic agents [Knuefermann et al., 2003]. In addition, transfection of ER-positive human breast cancer cells with the HER2 gene results in tamoxifen resistance [Benz et al., 1993], an effect that may result from interactions between the estrogen receptor and the HER2 receptor [Shou et al., 2004; Osborne et al., 2005].

The IGF-1 receptor (IGF-1R) has been shown to play a major role in the initiation and progression of the oncogenic process [Baserga, 1995, 1999; Baserga and Morrione, 1999; Baserga et al., 2003]. In addition to the HER2 kinase, the estrogen receptor also interacts with the IGF-1R [Lee et al., 1997; Yee and Lee, 2000; Martin and Stoica, 2002]. Thus, an agent that targets both HER2 and IGF-I receptors could have therapeutic potential in the treatment of tumors overexpressing HER2 as well as in overcoming or preventing resistance to therapy with anti-hormonal agents.

The National Cancer Institute has documented that the compound nordihydroguaiaretic acid (NDGA) inhibited the growth of all eight human breast cancer cell lines tested in their panel (http://dtp.nci.nih.gov/index.html). Recently, we reported that NDGA inhibits HER2 and IGF-1R activities in vitro and suppresses the growth of mammary tumors in mice [Youngren et al., 2005]. In the present study, we have evaluated the effect of this compound on a HER2-overexpressing, tamoxifen resistant breast cancer cell line (MCF-7/HER2-18) [Benz et al., 1993].

MATERIALS AND METHODS

Materials

NDGA, IGF-1, and IGF binding protein-3 (IGFBP-3) were generously provided by Insmed, Inc. (Glen Allen, VA). Antibodies against the IGF-1R (C-20), HER2 (C-18), and phosphospecific antibodies recognizing phosphotyrosine (PY20), and pNeu (Tyr1248), and HRP-conjugated anti-phosphotyrosine antibody (PY20HRP) were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Alpha IR3, a monoclonal antibody against the IGF-1R, was obtained from CalBiochem (San Diego, CA). Phosphospecific antibodies pIGF-IR (Y1131) and pAkt(ser473) were obtained from Cell Signaling (Beverly, MA). All other reagents were from Sigma (St. Louis, MO), except as indicated below. Gefitinib (Iressa) was a gift from Mark Moasser (University of California, San Francisco). Herceptin was purchased from a commercial pharmacy.

Cell Culture

MCF-7 cells stably transfected with the full length HER2 cDNA (MCF-7/HER2-18) or control vector (MCF-7/neo) were generously provided by Dr. Christopher Benz (Buck Institute for Age Research, Novato, CA) and were maintained at 37°C, 5%CO_2 in DMEM + 10% FCS (DMEM-10) supplemented with 200 μg/ml Geneticin.

Preparation of Cell Lysates

For dose effects of RTK inhibitors (NDGA or gefitinib) on cellular IGF-1R and HER2 signaling, cells were grown in six-well plates to ~80% confluency, then serum-starved for 18 h. RTK inhibitors were dissolved in DMSO and diluted with culture medium before being added to cells for 1.5 h at 37°C. The final concentration of DMSO during the incubation was 0.3%. For some studies, cells were also stimulated with 3 nM IGF-I for 10 min at 37°C. Reactions were terminated by rapidly aspirating medium and washing cells three times with ice cold PBS. Cells were harvested and solubilized in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 2 mM vanadate for 1 h at 4°C. Protein concentrations were determined by BCA assay (Pierce, Rockford, IL).

Enzyme Linked Immunosorbent Assays (ELISA) for Phosphorylated IGF-1R and HER2

IGF-1R phosphorylation was determined by ELISA as described previously for the insulin receptor [Youngren et al., 1997]. Briefly, 10 μg lysate protein were added to triplicate wells in a 96-well plate coated with monoclonal antibody to the IGF-1R (IR3; 2 μg/ml), and incubated for 18 h at 4°C. Plates were washed five times and then HRP-conjugated anti-phosphotyrosine antibody (0.3 μg/ml), diluted in Solution B (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.05% Tween-20, 1 mM PMSF, 2 mM vanadate, and 1 mg/ml bacitracin), was added for 2 h at 22°C. Plates were washed five times prior to color development with TMB substrate, which was terminated with 1.0 M H_3PO_4. Values for

receptor phosphorylation were determined by measuring absorbance at 450 nm.

HER2 phosphorylation was also determined by ELISA as above, using 2 μg of lysate protein per well and 2 μg/ml Herceptin as the capturing antibody.

Western Blot Analysis

Total protein extracts (10 μg), prepared as described above from cells cultured in the presence or absence of IGF-1 and/or RTK inhibitors, were subjected to SDS–PAGE and subsequently transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with primary antibody diluted in Superblock (Pierce) containing 0.1% Tween-20 (Bio-Rad). The membranes were washed three times with TBS + 0.05% Tween-20, then incubated with HRP-conjugated secondary antibody diluted in Superblock/Tween-20 for 90 min at room temperature. Membranes were washed again and bound antibodies detected by enhanced chemiluminescence (Pierce). Primary antibodies against the following proteins were used at the indicated dilutions: IGF-1R used at 0.2 μg/ml, HER2 used at 0.2 μg/ml, p-IGF-1R (Y1131) used at 1:1,000, pNeu (Tyr1248) used at 0.2 μg/ml, and pAkt(ser473) used at 1:1,000. Secondary HRP-conjugated antibodies were directed against the appropriate species of origin of the primary antibody.

Cell Growth Assays

The inhibitory effects of gefitinib, IGFBP-3, or NDGA on breast cancer cell growth were determined using a CyQuant cell proliferation assay kit (Molecular Probes, Eugene, OR). MCF-7/neo or MCF-7/HER2-18 cells were plated in 96-well plates (4×10^3 cells/well) in 100 μl/well of DMEM-10 medium. Cells were allowed to adhere overnight and were then treated with various concentrations of gefitinib, IGFBP-3, NDGA, or DMSO as a vehicle control in 100 μl/well of serum free DMEM (SF-DMEM), making the final serum concentration 5%. Media with inhibitors was refreshed on day 3 and the cultures were harvested on day 6. The plates were inverted onto paper towels with gentle blotting to remove growth medium without disrupting the adherent cells. Each plate was kept at −80°C until assayed for cell growth. After thawing the plate at room temperature, 200 μl of CyQuant GR solution was added to each well and the plates were incubated in the dark for 5 min. Fluorescence was measured with a SpectraMax Gemini XS fluorescence microplate reader (Molecular Devices) with 480-nm excitation and 520-nm emission.

The growth inhibitory effects of tamoxifen, in the presence or absence of NDGA, were assessed using a CyQuant assay as described above with a few modifications. Cells were estrogen-starved for 3 days in DMEM containing 10% charcoal-dextran-stripped FCS (CDSS; HyClone, Logan, UT) prior to their plating in 96-well plates. Cells were plated in 100 μl of the same media, allowed to adhere overnight, and then were switched to DMEM + 10%CDSS supplemented with 100 pM estrogen. Tamoxifen (100 nM final concentration) and/or NDGA (10–20 μM final concentration) was added in 100 μl of SF-DMEM to yield a final concentration of 5% CDSS in DMEM. The media and inhibitors were refreshed on day 3, the cultures were harvested on day 6, and a CyQuant assay was performed as described above.

Cell Cycle Analysis

MCF-7/neo and MCF-7/HER2-18 cells were treated with 30 μM NDGA (or DMSO as a vehicle control) for 24 h. Adherent and non-adherent cells were harvested, washed twice with PBS containing 1% glucose (PBS/G), and then fixed in ice-cold 70% ethanol overnight at 4°C. After fixing, the cells were pelleted by centrifugation and the cell pellets were resuspended in propidium iodide (PI) staining solution (50 μg/ml PI, 100 U/ml RNaseA, in PBS/G). These samples were incubated at room temperature for at least 30 min and analyzed on a FACS Calibur flow cytometer within 24 h. The resulting histograms were analyzed for cell cycle distribution using ModFit software (BD Biosciences, San Jose, CA).

Induction of Apoptosis

To assay for DNA fragmentation as a marker of apoptosis, we used a procedure for the isolation of genomic DNA that allows for the analysis of both high and low molecular weight DNA fragmentation during apoptosis [Solovyan and Salminen, 1999]. MCF-7/neo and MCF-7/HER2-18 cells were plated at 5×10^5 cells/well in six-well plates and allowed to adhere overnight. NDGA (30 μM in DMSO) was added to duplicate wells for each cell line. DMSO was added to duplicate control wells. After 24 h,

attached and floating cells were harvested, washed in cold PBS, and embedded in agarose droplets [Solovyan and Salminen, 1999]. The agarose droplets containing cells were incubated in lysis buffer (50 mM NaCl, 20 mM Tris–HCl, pH 8.0, 20 mM EDTA, 0.5% sodium sarkosyl, 50 μg/ml RNaseA, and 100 μg/ml proteinase K) for 1 h at 37°C, then washed, inserted into the wells of a conventional 1% agarose gel, and electrophoresed overnight at ~1 V/cm. The ethidium bromide stained gels were photographed under UV transillumination and inverse images are presented.

RESULTS

HER2 Receptor, but Not the IGF-1R, Is Overexpressed in MCF-7/HER2-18 Cells

Various studies have demonstrated reduced IGF-1R expression in antiestrogen-resistant cell lines [McCotter et al., 1996; van den Berg et al., 1996; Brockdorff et al., 2003; Frogne et al., 2005]. We examined the levels of the IGF-1R and HER2 proteins in parental MCF-7/neo and MCF-7/HER2-18 cells using Western blot analyses. Compared to the MCF-7/neo cells, the level of IGF-1R was slightly decreased in MCF-7/HER2-18 cells (Fig. 1), consistent with the reports described above.

In contrast, the HER2 protein was abundant in the MCF-7/HER2-18 cells, but expressed at a very low level in MCF-7/neo cells (Fig. 1). This observation is in agreement with the original report that MCF-7/HER2-18 cells overexpress the HER2 protein compared to parental MCF-7 cells [Benz et al., 1993].

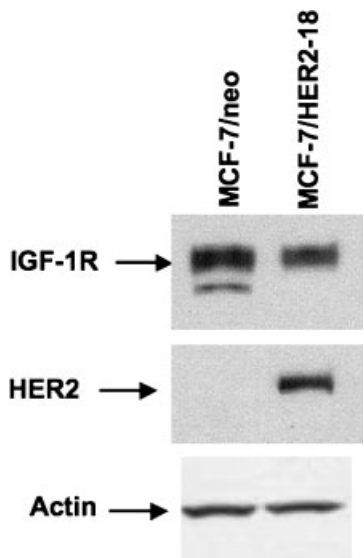

Fig. 1. Expression of IGF-IR and HER2 in MCF-7/neo and MCF-7/HER2-18 cells. Cell lysates were separated by SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies specific for IGF-1R, HER2, and actin.

Differential Growth Inhibitory Effects of Gefitinib, IGFBP-3, and NDGA

Gefitinib, an RTK inhibitor that blocks signaling through the EGFR and HER2 receptors but not the IGF1-R, was more effective at inhibiting the growth of HER2-overexpressing MCF-7/HER2-18 cells when compared to MCF-7/neo cells (Fig. 2A). In contrast, IGFBP-3, a protein that blocks IGF1-R but not HER2 signaling, had a greater growth inhibitory effect on MCF-7/neo cells compared to MCF-7/HER2-18 cells (Fig. 2B). Finally, NDGA, a RTK inhibitor that blocks signaling through both the IGF1-R and HER2, was equally effective in both cells lines, with a one-half maximal effect occurring at 12.5 μM (Fig. 2C).

NDGA Inhibits IGF-1R and HER2 Kinase Activity in MCF-7/HER2-18 Cells

The tamoxifen resistant MCF-7/HER2-18 cell line expresses both IGF-1R and HER2, two RTK that play a role in breast cancer. We have previously shown that NDGA inhibits the kinase activities of IGF-1R in MCF-7 cells and HER2 in SKBR-3 cells [Youngren et al., 2005]. We next compared the effects of NDGA with gefitinib on the activities of these receptor kinases in MCF-7/HER2-18 cells.

Employing a sensitive ELISA [Youngren et al., 1997], we observed that gefitinib had only a weak inhibitory effect on IGF-1 stimulated phosphorylation of the IGF-1R (Fig. 3A). In contrast, gefitinib significantly inhibited HER2 phosphorylation with a half maximal effect occurring at less than 5 μM (Fig. 3A). Whereas gefitinib suppressed HER2 but not IGF-1R activity, NDGA strongly inhibited both kinases as measured by phosphotyrosine specific ELISAs (Fig. 3B) and Western blot analyses (Fig. 3B, inset). The Western blots also demonstrate that the inhibition of kinase activity is not due to a reduction in the amount of HER2 or IGF-1R expression.

NDGA Arrests Cells in the S Phase of the Cell Cycle and Induces Apoptosis

To determine if the growth inhibition induced by NDGA involved cell cycle changes, we examined cell cycle phase distribution of breast

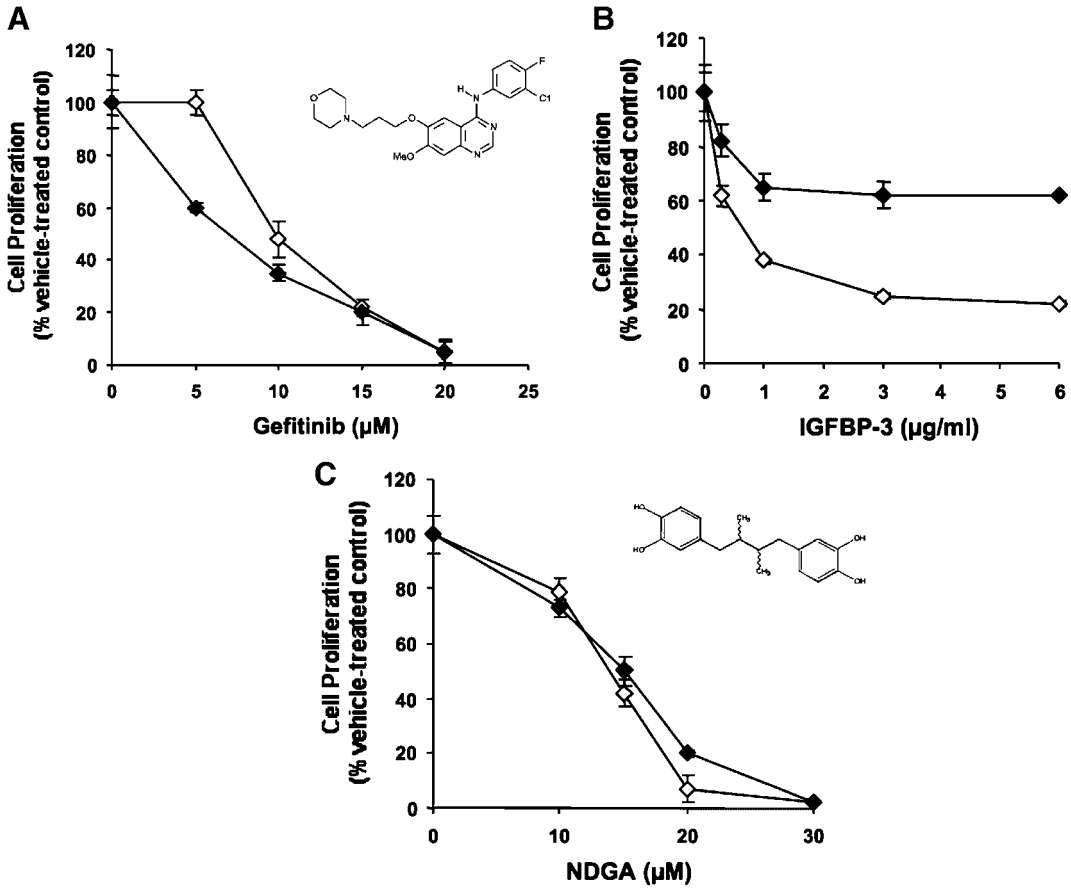

Fig. 2. Effects of gefitinib, IGFBP-3, and NDGA on the growth of MCF-7/neo (open symbols) and MCF-7/HER2-18 cells (closed symbols). Cells were grown in the presence of various concentrations of gefitinib (**A**), IGFBP-3 (**B**), or NDGA (**C**) for 6 days. Cell growth was assessed with a CyQuant cell proliferation assay. The results are expressed as a percentage of vehicle (DMSO) treated control cells (mean ± SEM of triplicate wells) and are representative of three separate experiments. The chemical structures of gefitinib and NDGA are depicted on **panels A**,**C**, respectively.

cancer cells treated with NDGA (or DMSO as a vehicle control) using PI staining and flow cytometric analyses. As shown in Figure 4, treatment of MCF-7/neo cells with NDGA for 24 h increased the proportion of cells in S phase from 39.1% (panel A) to 53.7% (panel B). Similarly, NDGA treatment of MCF-7/HER2-18 cells increased the proportion of cells in S phase from 45.1% (panel C) to 61.7% (panel D).

Apoptotic cells can be recognized by their diminished stainability with the DNA-specific fluorochrome PI measured using flow cytometry. An apoptotic/hypodiploid population (sub-G1 peak) can be quantified by DNA content frequency histograms. As shown in Figure 4, under control conditions (DMSO treatment), the apoptotic populations (sub-G1 peak) of MCF-7/neo and MCF-7/HER2-18 cells were 8.7% and 8.2%, respectively (Fig. 4, panels A,C). Treatment with NDGA increased the proportion of apoptotic MCF-7/neo and MCF-7/HER2-18 cells to 25.3% and 32.6%, respectively (Fig. 4, panels B,D).

To confirm the apoptosis inducing effects of NDGA, we performed assays for DNA fragmentation. MCF-7/neo and MCF-7/HER2-18 cells were plated and treated with 30 μM NDGA for 24 h. Attached and floating cells were harvested, washed in cold PBS, embedded in agarose droplets and processed for gel electrophoresis as described in Materials and Methods Section. The results of this experiment are shown in Figure 5. Control cells (incubated with DMSO) demonstrated a slight amount of DNA fragmentation, but the majority of the intact genomic DNA remained in the well (Fig. 5, lanes 1,3). The weak bands of DNA that entered the gels in the control lanes probably reflect a small percentage of dying cells in culture. In contrast, NDGA induced high molecular weight (HMW) DNA

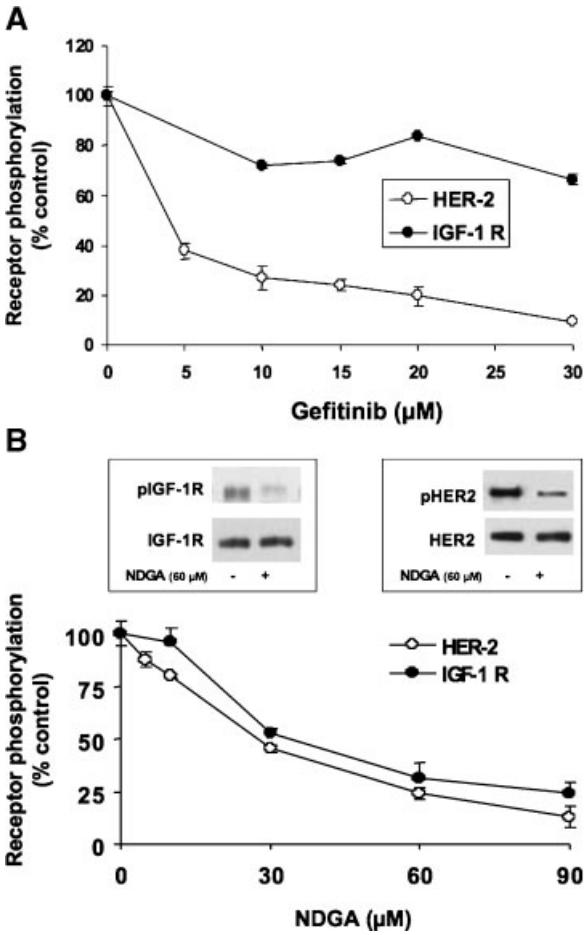

Fig. 3. Effects of gefitinib and NDGA on IGF-IR and HER2 phosphorylation in MCF-7/HER2-18 cells. Cells treated with various concentrations of gefitinib (**A**) or NDGA (**B**) were lysed and assayed for IGF-1R and HER2 phosphorylation by ELISA. For the IGF-1R ELISA, cells were stimulated with 3 nM IGF-I for 10 min. For the HER2 ELISA, cells were not stimulated. The results are expressed as mean ± SEM of triplicate wells and are representative of three separate experiments. The effects of NDGA were confirmed by Western blot (**panel B**, inset) where phosphorylated IGF-IR and HER2 were detected with phospho-specific antibodies to pIGF-IR and pHER2, respectively.

fragmentation as evidenced by a significant fraction of the DNA entering the gel but not being resolved and forming a compression band near the top of the gel (Fig. 5, lanes 2,4). Taken together, these results suggest that NDGA inhibited the proliferation of MCF-7/neo and MCF-7/HER2-18 cells via S phase arrest of the cell cycle leading to apoptosis.

NDGA Inhibits Signaling Through AKT

The serine kinase AKT/PKB is activated by RTK, including IGF-1R and HER2, and mediates cell growth and survival [Ahmad et al., 1999; Martin et al., 2000; Stoica et al., 2003; Mitsiades et al., 2004]. We measured the effects of NDGA on the phosphorylated state of this protein in MCF-7/HER2-18 cells. In the absence of IGF-1, AKT/PKB was phosphorylated and the level of phospho-AKT/PKB was increased by the addition of 3 nM IGF-1 (Fig. 6). NDGA inhibited both the basal and the IGF-1 stimulated phosphorylation of AKT/PKB (Fig. 6). Treatment with NDGA did not alter the content of the AKT/PKB protein in MCF-7/HER2-18 cells.

NDGA and Tamoxifen Show Additive Growth Inhibition Against MCF-7/HER2-18 Cells

Several reports have demonstrated interactions between IGF-1R and HER2 signaling pathways [Lu et al., 2004; Gee et al., 2005; Nahta et al., 2005] as well as between ER signaling and these RTKs [Lee et al., 1997; Yee and Lee, 2000; Martin and Stoica, 2002]. Given these interactions, we examined whether NDGA (which inhibits both HER2 and IGF-1R activities) could overcome tamoxifen resistance in MCF-7/HER2-18 cells.

Tamoxifen at 100 nM inhibited the growth of MCF-7/neo cells by over 50% (Fig. 7A). In contrast, tamoxifen at 100 nM had less of an effect on MCF-7/HER2-18 cells (<25% growth inhibition) (Fig. 7B), consistent with other reports demonstrating tamoxifen resistance of MCF-7/HER2-18 cells [Benz et al., 1993].

Both NDGA and tamoxifen had antiproliferative effects on the tamoxifen sensitive MCF-7/neo cells (Fig. 7A). NDGA at 10 and 15 μM inhibited growth by 23% and 55%, respectively. However, when combined, NDGA treatment did not enhance the growth inhibitory effects of tamoxifen in these cells (Fig. 7C). In contrast to MCF-7/neo cells, NDGA treatment significantly enhanced the antiproliferative effects of tamoxifen in the anti-estrogen resistant MCF-7/HER2-18 cells (Fig. 7D). NDGA alone, at 10 and 15 μM, inhibited the growth of MCF-7/HER2-18 cells by 9 and 38%, respectively. While tamoxifen alone induced a 24% reduction in growth, the combination of tamoxifen with 10 or 15 μM NDGA resulted in 40 and 60% growth inhibition, respectively, indicating additive effects of NDGA and tamoxifen.

DISCUSSION

Interference with growth factor signals that drive cell proliferation and survival is an attractive strategy for cancer treatment. Initial

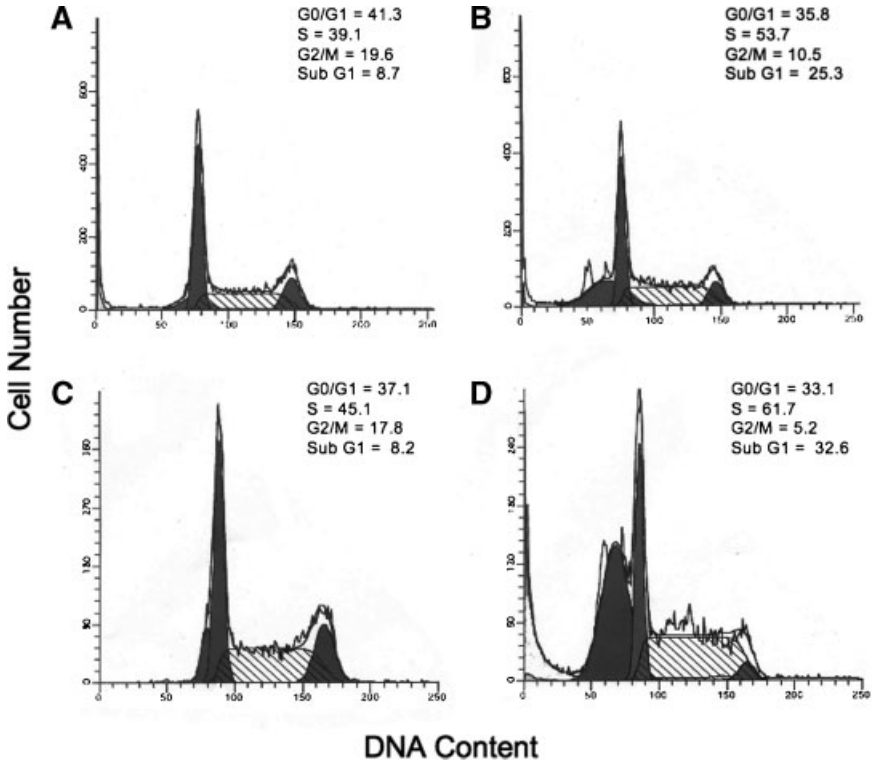

Fig. 4. Effect of NDGA on cell cycle distribution and apoptosis. MCF-7/neo cells (**A**,**B**) and MCF-7/HER2-18 cells (**C**,**D**) were incubated for 24 h with 30 μM NDGA (B,D) or with DMSO vehicle control (A,C). Cells were then harvested and cell cycle distribution was analyzed by flow cytometry. The percentages of cells distributed in G0/G1, S, G2/M, and sub-G1 regions of the histograms are indicated.

explorations have concentrated on types of cancers in which growth factor signaling is elevated and plays a dominant role in driving cell proliferation. A well known example is breast cancer with overexpression of the HER2 receptor which responds to interventions that block HER2 action, such as gefitinib [Benz et al., 1993; Arteaga et al., 2002; Herbst and Kies, 2003; Konecny et al., 2003; Arteaga and Truica, 2004; Kaklamani and O'Regan, 2004; Von Pawel, 2004; Agrawal et al., 2005; Johnston, 2005; Penne et al., 2005; Wakeling, 2005] or Herceptin [Slamon et al., 2001; Vogel et al., 2002; Baselga et al., 2005; Marty et al., 2005]. Herein we have examined the action of NDGA, a RTK inhibitor that blocks signaling through HER2 receptors, as well as IGF-1Rs [Youngren et al., 2005], and compared it with that of gefitinib, which is directed at EGFR/HER2 receptors, or IGFBP-3, which is directed at the IGF1-R.

We studied the actions of these drugs on MCF-7/neo cells, an estrogen receptor positive human breast cancer cell line that is sensitive to the antiestrogen tamoxifen, and MCF-7/HER2-18 cells that overexpress the HER2 receptor and demonstrate a reduced sensitivity to tamoxifen. MCF-7/neo cells express IGF-1Rs but only low levels of HER2, whereas the MCF-7/HER2-18 cell line expresses IGF-1R and high levels of HER2. Our studies showed that gefitinib, as expected from its mode of action, inhibited the growth of MCF-7/HER2-18 cells, but had less potency on MCF-7/neo cells. These observations are consistent with the notion that inhibition of HER2 receptors is likely to be most effective on cells whose proliferation is driven by enhanced activity of this receptor pathway. IGFBP-3 on the other hand was more effective against the MCF-7/neo cells compared to the MCF-7/HER2-18 cells. IGFBP-3 is a member of a family of proteins that interact with IGFs and modulate their availability and interaction with IGF receptors [Pratt and Pollak, 1994; Figueroa et al., 1995; Oh et al., 1995; Baxter, 2001; Jerome et al., 2006]. IGF binding proteins have been demonstrated to reduce cellular prolife-

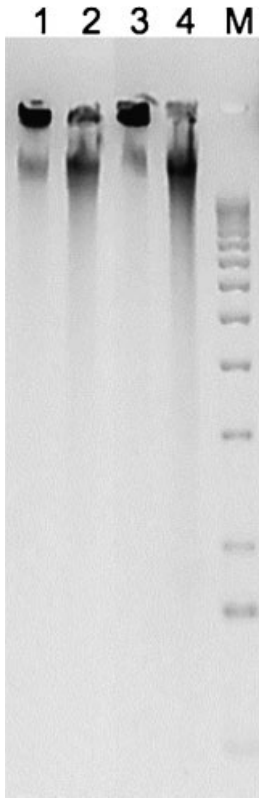

Fig. 5. Patterns of nuclear DNA fragmentation during apoptosis induced by NDGA. MCF-7/neo cells (**lanes 1**,**2**) and MCF-7/HER2-18 cells (**lanes 3**,**4**) were incubated for 24 h with 30 μM NDGA (lanes 2,4) or with DMSO vehicle control (lanes 1,3). The cells were then collected, embedded into ''agarose drops,'' lysed, and fractionated by conventional agarose gel electrophoresis.

ration and induce apoptosis in a variety of cancer cell lines in vitro [Pratt and Pollak, 1994; Figueroa et al., 1995; Oh et al., 1995; Baxter, 2001; Jerome et al., 2006].

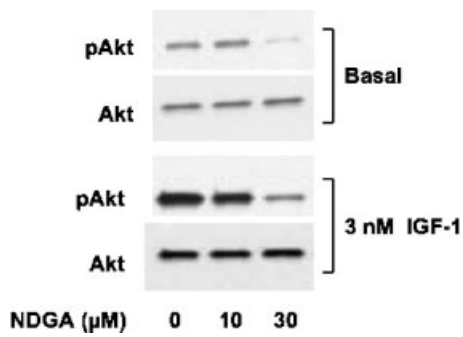

Fig. 6. Effect of NDGA on Akt/PKB phosphorylation. MCF-7/HER2-18 cells were incubated in the presence or absence of 3 nM IGF-1 for 10 min, with or without NDGA treatment. Cells lysates were prepared and separated by SDS–PAGE, transferred to nitrocellulose membranes, and probed with an anti-pAkt antibody to detect phosphorylated Akt protein. These blots were subsequently stripped and re-probed with an anti-Akt antibody to detect total Akt protein.

In contrast to the selective efficacy of gefitinib and IGFBP-3, we found that NDGA is equally effective at inhibiting cell proliferation of both MCF-7/neo cells and MCF-7/HER2-18 cells. The kinetics of inhibition of cell proliferation of MCF-7/HER2-18 cells match those of NDGA inhibition of IGF-1R phosphorylation with half-maximal effects in the range of 10–20 μM. Inhibition of HER2 receptor phosphorylation was also evident, with half-maximal effects in the range of 30 μM.

Inhibition of MCF-7/neo and MCF-7/HER2-18 cells by NDGA is accompanied by an arrest of cells in the S phase of the cell cycle, the induction of apoptosis, and a decrease in downstream signaling (Akt phosphorylation). Similar to our results, NDGA has been demonstrated to induce an S phase cell cycle arrest in other cancer cell lines [Soriano et al., 1999; Hofmanova et al., 2002]. Our flow cytometry analyses of NDGA treated breast cancer cells also revealed a sub-G1 peak, suggesting the presence of apoptotic cells. To further study apoptosis induction by NDGA, assays for DNA fragmentation were performed using a procedure for the isolation of genomic DNA that allows for the analysis of both high and low molecular weight DNA fragmentation during apoptosis [Solovyan and Salminen, 1999]. Low molecular weight DNA ladders are produced in some apoptotic cells but not others, whereas nearly all cells undergoing apoptosis display HMW DNA fragmentation [Walker and Sikorska, 1997]. Although these HMW DNA fragments are too large to be resolved by conventional gel electrophoresis, they are able to leave the wells and enter conventional agarose gels, forming a compression band of about 20–50 kb, the upper limit of fractionation ability of these gels [Walker et al., 1993; Solovyan and Salminen, 1999]. NDGA induced HMW DNA fragmentation in both MCF-7/neo and MCF-7/HER2-18 cells suggesting that the growth inhibitory activity of this compound is via an apoptotic mechanism. We have also demonstrated apoptosis induction in these cell lines by annexin V staining (data not shown).

Susin et al. [2000] have described two distinct parallel pathways leading to chromatin processing during apoptosis. One pathway involves the caspase activator Apaf-1, caspases, and caspase-activated DNAse (CAD). This pathway results in oligonucleosomal DNA fragmentation and advanced chromatin condensation.

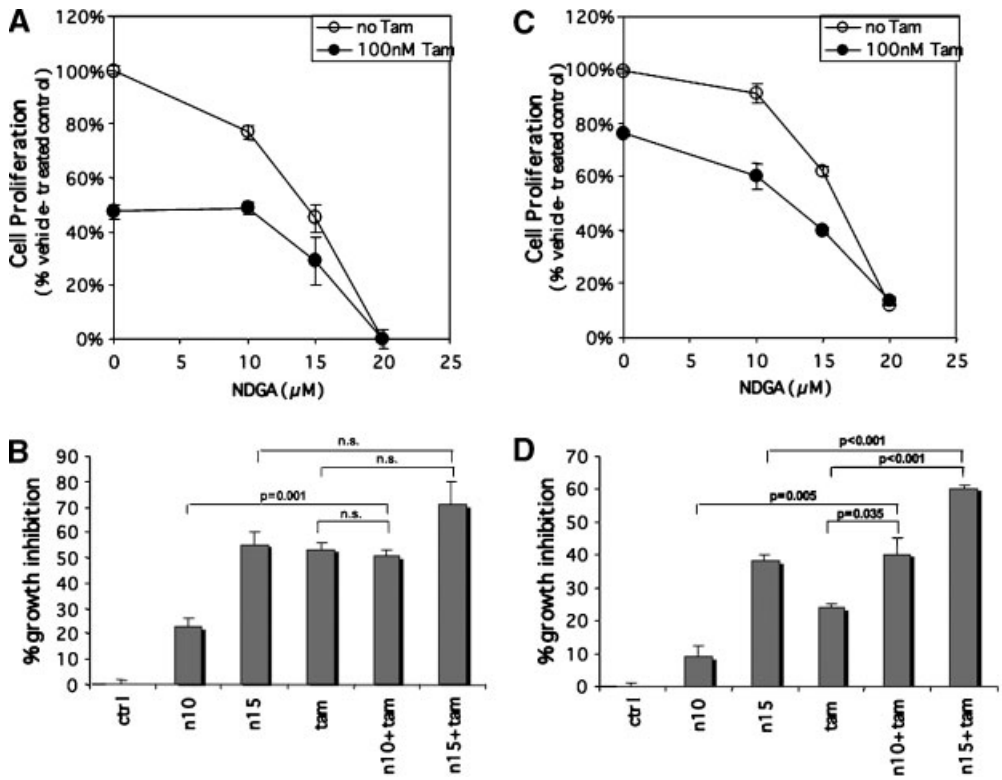

Fig. 7. Effects of combined treatment with tamoxifen and NDGA on the growth of MCF-7/neo and MCF-7/HER2-18 cells. MCF-7/neo (**panels A**,**C**) and MCF-7/HER2-18 (**panels B**,**D**) cells were incubated for 6 days with 100 nM tamoxifen, in the presence or absence of various concentrations of NDGA and cell growth was assessed with a CyQuant assay. Cell proliferation was expressed as a percentage of vehicle-treated control cells (mean ± SEM) (panels A,B). Panels C,D express the results as percent growth inhibition. n10, n15, and tam indicate treatment with 10 and 15 μM NDGA and 100 nM tamoxifen, respectively. n10 + tam and n15 + tam indicate treatment with 10 μM NDGA + 100 nM tamoxifen and 15 μM NDGA + 100 nM tamoxifen, respectively. Data were analyzed using Student's *t*-test and the resulting *P*-values are shown; n.s. indicates not significant ($P > 0.05$).

The second pathway is caspase-independent and involves translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus. This leads to large scale DNA fragmentation and peripheral chromatin condensation, but does not result in oligonucleosomal DNA fragmentation. NDGA appeared to induce only large scale fragmentation, suggesting that it is inducing apoptosis via the caspase-independent pathway.

Breast cancers are either estrogen-dependent or independent. A subset of breast cancers, despite the presence of estrogen receptors, do not respond to endocrine therapy and it has been reported that HER2 expression is associated with a reduced response rate to hormone therapy of metastatic breast cancer [Wright et al., 1989]. Transfection of ER-positive cells with a HER2 cDNA, resulting in overexpression of this RTK, also results in resistance to tamoxifen treatment [Benz et al., 1993]. In view of the disruption of tamoxifen action in HER2-overexpressing cells, studies have examined the effects of blocking HER2 signaling in tamoxifen resistant breast cancer cells. It has been shown that gefitinib can overcome tamoxifen resistance, or prevent its development, both in vitro and in a mouse xenograft model [Kurokawa and Arteaga, 2001; Gee et al., 2003; Shou et al., 2004]. However, continuous treatment eventually leads to acquired resistance to gefitinib which is associated with increased signaling via the IGF-1R [Jones et al., 2004].

We examined the interaction of NDGA (which inhibits both IGF-1R and HER2) with tamoxifen on the growth of tamoxifen resistant MCF-7/HER2-18 cells. NDGA alone inhibited the proliferation of both tamoxifen sensitive MCF-7/neo and tamoxifen resistant MCF-7/HER2-18 cells. Notably, NDGA combined with tamoxifen demonstrated additive growth inhibitory effects on MCF-7/HER2-18 cells. The development of acquired resistance to anti-hormonal therapies such as tamoxifen is a major therapeutic

problem in breast cancer. These results suggest that NDGA might be clinically useful, in conjunction with anti-hormonal agents, in the treatment of hormone-resistant breast cancer, or possibly in preventing the development of acquired resistance to these agents.

NDGA has been administered to cancer patients in limited clinical trials. Dunphy et al. [2004] administered a methylated version of NDGA (tetra-*o*-methyl NDGA; M4N) via intratumoral injection in three patients with refractory head and neck cancers. Two of these patients were found to have total necrosis of the injected tumor site. However, their tumors continued to grow outside the boundaries of these necrotic areas. Human prostate cancer cells have been shown to express IGF-1Rs [Ryan et al., in press]. Accordingly, a phase I clinical trial was recently completed studying the side effects and best dose of NDGA in treating patients with nonmetastatic relapsed prostate cancer (www.clinicaltrials.gov identifier: NCT00313534). Oral doses of up to 2.5 g/day, administered for 28 days, were well tolerated (Charles Ryan, UCSF, personal communication). In addition, clinicaltrials.gov lists two ongoing trials of tetra-*o*-methyl NDGA (EM-1421). One is an NCI sponsored phase I/II trial studying the side effects and best dose of EM-1421 in patients with recurrent high-grade glioma (NCT00404248). The second is a phase I, dose escalation study, sponsored by Erimos Pharmaceuticals, to determine the safety and maximum tolerated dose of EM-1421 given by intravenous infusion.

In summary, we demonstrated that NDGA inhibits the kinase activities of the IGF-1R and the HER2 receptor, leading to an S phase cell cycle arrest, growth inhibition, and the induction of apoptosis in MCF-7/neo and MCF-7/HER2-18 cells. In addition, NDGA combined with tamoxifen demonstrated additive growth inhibitory effects against the tamoxifen resistant, HER2-overexpressing MCF-7/HER2-18 cell line. These data raise the possibility that NDGA, a RTK inhibitor, may have a broad spectrum of action on breast cancers with a variety of perturbations in their signaling pathways.

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