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Nordihydroguaiaretic Acid Inhibits Insulin-Like Growth Factor Signaling, Growth, and Survival in Human Neuroblastoma Cells

Gary E. Meyer,[1] Louis Chesler,[1] Dandan Liu,[1] Karissa Gable,[2] Betty A. Maddux,[2] David D. Goldenberg,[3] Jack F. Youngren,[2] Ira D. Goldfine,[2] William A. Weiss,[3] Katherine K. Matthay,[1] and Stephen M. Rosenthal[1]*

[1]Department of Pediatrics, University of California, San Francisco, California
[2]Diabetes and Endocrine Research, University of California, San Francisco/Mt. Zion Medical Center, San Francisco, California
[3]Department of Neurology, University of California, San Francisco, California

Abstract Neuroblastoma is a common pediatric malignancy that metastasizes to the liver, bone, and other organs. Children with metastatic disease have a less than 50% chance of survival with current treatments. Insulin-like growth factors (IGFs) stimulate neuroblastoma growth, survival, and motility, and are expressed by neuroblastoma cells and the tissues they invade. Thus, therapies that disrupt the effects of IGFs on neuroblastoma tumorigenesis may slow disease progression. We show that NVP-AEW541, a specific inhibitor of the IGF-I receptor (IGF-IR), potently inhibits neuroblastoma growth in vitro. Nordihydroguaiaretic acid (NDGA), a phenolic compound isolated from the creosote bush (*Larrea divaricata*), has anti-tumor properties against a number of malignancies, has been shown to inhibit the phosphorylation and activation of the IGF-IR in breast cancer cells, and is currently in Phase I trials for prostate cancer. In the present study in neuroblastoma, NDGA inhibits IGF-I-mediated activation of the IGF-IR and disrupts activation of ERK and Akt signaling pathways induced by IGF-I. NDGA inhibits growth of neuroblastoma cells and induces apoptosis at higher doses, causing IGF-I-resistant activation of caspase-3 and a large increase in the fraction of sub-G_0 cells. In addition, NDGA inhibits the growth of xenografted human neuroblastoma tumors in nude mice. These results indicate that NDGA may be useful in the treatment of neuroblastoma and may function in part via disruption of IGF-IR signaling.
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Key words: insulin-like growth factor I receptor; neuroblastoma; small molecule inhibitor; nordihydroguaiaretic acid; apoptosis

Neuroblastoma affects an estimated 1 in 7,000 children under age 15 [Carlsen, 1992]. Children with metastatic disease have a less than 50% chance of survival [Goldsby and Matthay, 2004]. Neuroblastoma tumors are believed to arise from neural crest cells in the adrenal gland and spinal ganglia [Castleberry, 1997; Cianfarani and Rossi, 1997]. Neuroblastoma often regresses spontaneously in children under 1 year of age [Brodeur et al., 1997; Cianfarani and Rossi, 1997; Nakagawara, 1998], but more aggressive disease in older children is difficult to treat with conventional radiation and chemotherapies [Philip, 1992]. Metastasis to bone, meninges, the liver, and other organs contributes to the difficulty in eliminating the disease [Philip, 1992; Tanabe et al., 1995; DuBois et al., 1999], and children in this group have only a 20–35% chance of survival with currently available treatments [Goldsby and Matthay, 2004]. The development of effective treatments for neuroblastoma is hampered by an incomplete understanding of

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*Correspondence to: Stephen M. Rosenthal, MD, Division of Endocrinology, Department of Pediatrics, University of California, San Francisco, S672 513 Parnassus Ave., San Francisco, CA 94143. E-mail: rosenthals@peds.ucsf.edu

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the factors that lead to neuroblastoma tumorigenesis.

Growth factor responsiveness is believed to promote tumor growth, survival, and invasiveness. Therapeutic approaches that disrupt growth factor signaling may impact disease progression. Nordihydroguaiaretic acid (NDGA), a naturally occurring compound isolated from creosote (*Larrea divaricata*), inhibits the activation of partially purified insulin-like growth factor I (IGF-I) and her2/neu receptor tyrosine kinases [Youngren et al., 2005]. In breast cancer cells, NDGA inhibits ligand activation of the IGF-I and her2/neu receptors and subsequent activation of signaling intermediates downstream of these receptors [Youngren et al., 2005]. Both the in vitro and in vivo growth of breast cancer cells is inhibited by NDGA, potentially via its ability to suppress responsiveness to growth factors [Youngren et al., 2005].

Neuroblastoma cells are particularly sensitive to IGF-I and II, peptide growth factors that stimulate mitogenesis and survival. IGFs bind to the tyrosine kinase IGF-I receptor (IGF-IR), causing receptor autophosphorylation and activation of the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI-3K) signaling pathways. MAPK regulates mitogenesis [De Meyts et al., 1994], while PI-3K activates targets that impact apoptosis, such as Akt [Fresno Vara et al., 2004].

IGFs promote neuroblastoma tumorigencity by stimulating proliferation, inhibiting apoptosis, and stimulating motility. IGFs are expressed in all neuroblastoma tumor stages and in neuroblastoma tumor lines [Martin et al., 1992; Sullivan et al., 1995], and can act as either autocrine or paracrine mitogens [Martin and Feldman, 1993; Meghani et al., 1993; Leventhal et al., 1995; Kiess et al., 1997]. IGF-I and IGF-IR expression prevent neuroblastoma cells from undergoing apoptosis by regulating the activity of caspase-3 [Singleton et al., 1996a,b; Matthews et al., 1997; Van Golen and Feldman, 2000; Van Golen et al., 2000]. IGFs also regulate the metastatic capabilities of neuroblastoma cells by stimulating actin polymerization, lamellipodium extension, and motility [Leventhal et al., 1997; Kim and Feldman, 1998; Meyer et al., 2001, 2005].

Given the effects of the IGFs on neuroblastoma tumorigenesis, drug interventions that interrupt IGF signaling may impair disease progression. A highly specific inhibitor of IGF-IR phosphorylation, NVP-AEW541, has anti-tumor affects in numerous cell lines [Garcia-Echeverria et al., 2004]. While the current manuscript was under review, NVP-AEW541 was shown to have potent anti-tumor effects against several neuroblastoma cell lines in vitro and in vivo [Tanno et al., 2006]. However, NVP-AEW541 is not expected to proceed to clinical trials. Even though NDGA likely affects multiple growth factor receptors, it is effective at inhibiting breast cancer growth, has anti-IGF-IR activity that could be effective against neuroblastoma, and is nearing completion of a phase I dose-escalation study in prostate cancer patients with no apparent dose limiting toxicities. Thus, NDGA is an attractive compound to consider for treatment of neuroblastoma. In this study we investigate the impact of NDGA on IGF signaling and tumorigenesis in neuroblastoma. We used SH-SY5Y, Kelly, and SHEP human neuroblastoma cell lines. The first two cell lines are highly aggressive and express high levels of IGF-IR. SHEP cells are not inherently tumorigenic and express little IGF-IR, but are still dependent upon IGFs for survival and thus serve as a model for IGF-dependent neuroblastoma cell lines with low IGF-IR expression. We quantified IGF-I- and serum-dependent growth of neuroblastoma cells treated with NVP-AEW541 and NDGA, as well as characterized IGF-I-dependent phosphorylation of IGF-IR, extracellular regulated kinases (ERKs), and Akt in the presence of NDGA. We found that NDGA inhibits IGF-IR activation and subsequent activation of MAPK and Akt. NDGA decreases proliferation, increases apoptosis, and decreases motility of neuroblastoma cells, and causes decreased xenograft tumor growth in vivo.

MATERIALS AND METHODS

Cell Culture and Reagents

Human SH-SY5Y, SHEP, and Kelly neuroblastoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% calf serum and maintained in a humidified incubator with 10% CO_2 at 37°C. NVP-AEW541 was a gift to WAW from Novartis Pharma AG (East Hanover, NJ). NDGA was a gift from Insmed Corporation (Richmond, VA). Caffeic acid, 5,8,11-eicosatriynoic acid (ETI), and cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) were purchased from Biomol International (Pilgrams

Landing, PA). NVP-AEW541, caffeic acid, ETI, CDC, and NDGA were dissolved immediately before each experiment in DMSO to make a 1,000× solution. IGF-I was purchased from GroPep (Adelaide, SA, Australia). Anti-IGF-IR antibody (αIR-3) was purchased from Calbiochem (San Diego, CA). Anti-insulin receptor antibody (MA-20) was developed in the Goldfine laboratory at the University of California, San Francisco. Anti-phosphotyrosine and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, anti-phospho-Akt, anti-ERK1/2, anti-phospho-ERK1/2, and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Horseradish peroxidase conjugated goat anti-rabbit IgG was purchased from Zymed Laboratories (South San Francisco, CA). CyQUANT was purchased from Molecular Probes (Eugene, OR). Propidium iodide was purchased from Sigma (St. Louis, MO).

IGF-IR and InsR Phosphorylation ELISAs

SH-SY5Y and SHEP cells were grown to 80% confluence in DMEM/10% calf serum, then serum-starved for 4 h. Cultures were then treated with DMSO or 60 μM NDGA and incubated for 1 h. Some cultures were then treated with 1 nM IGF-I for 10 min. The medium was removed, cultures rinsed 3× in cold PBS, and lysis buffer (120 mM HEPES, 300 mM NaCl, 2 mM sodium orthovanadate, and 1 mM phenylmethylsufonylfluoride (PMSF)) was added. Cultures were rocked in lysis buffer at 4°C for 1 h. Ninety six-well plates were coated with αIR-3 antibody (for IGF-IR) or MA-20 antibody (for Ins R) in 50 mM $NaHCO_3$, pH 9.0, for 2 h at RT. Plates were rinsed 3× in tris-buffered saline + 0.1% Tween (TBST), then blocked with SuperBlock (Pierce, Rockford, IL) for 30 min at RT. Each well of the ELISA plate received 30 μg of lysate protein from the cell cultures, followed by 24 h incubation at 4°C. Plates were rinsed 5× with TBST, and HRP-conjugated anti-phosphotyrosine antibody was added (1:2,000, diluted in 120 mM HEPES, 300 mM NaCl, 2 mM sodium orthovanadate, and 1 mM PMSF, 1% bovine serum albumin, 1 mg/ml bacitracin, and 0.5% Tween-20) for 2 h at RT. Plates were again rinsed 5× in TBST, and TMB (Pierce) was added until blue color was sufficiently developed. Absorbance at 451 nm was quantified. Each condition was performed in triplicate, and the experiment was repeated three times.

CyQUANT Assay for Cell Growth

Cells were plated on four 96-well tissue culture plates, in DMEM/10% calf serum at a density of 8,000 cells/well and incubated for 24 h. In one set of experiments, the medium was switched to serum free DMEM supplemented with 1% bovine serum albumin (to provide osmotic support). IGF-I (10 nM) was added to some samples. In a second set of experiments, the cells continued to be cultured in DMEM/10% calf serum for the duration of the experiment, with no additional IGF-I. For all experiments, DMSO or different concentrations of NVP-AEW541, CDC, caffeic acid, ETI, and/or NDGA were added to the cultures at 0 h (the day after plating). The range of doses used for each compound was based on their respective IC50s for the IGF-IR receptor (NVP-AEW541, NDGA) or lipoxygenases (caffeic acid, ETI, CDC). The media from one plate was immediately removed, and the plate was frozen at −80°C. This plate served as the baseline for the experiment. Single plates were frozen at 24, 48, and/or 72 h following addition of drugs. DNA content of each well was quantified by staining with CyQUANT according to the manufacturer's instructions and measuring CyQUANT absorbance with a fluorimeter. Each condition was performed with six replicates, and the experiment was repeated three times.

Detection of Phospho-Akt and Phospho-ERK

Cells were grown to 80% confluence, serum starved for 4 h, and treated with DMSO or different concentrations of NDGA for 1 h. Then, some cultures were treated with 10 nM IGF-I for 15 min. Cultures were immediately placed on ice, the medium was removed, and the cells were lysed in modified RIPA buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 1% deoxycholate). Fifty micrograms of protein from each sample were separated via SDS–PAGE and transferred to nitrocellulose. Phospho- and total-Akt and -ERK were detected by immunoblotting.

Propidium Iodide Staining of Apoptotic Cells

SH-SY5Y cells were cultured in DMEM with 10% calf serum. Cultures were treated with DMSO or NDGA for 24 h. The supernatant was

collected to save detached cells. Attached cells were removed from the plate via trypsinization, and pelleted by centrifugation in combination with the cells in the supernatant. The cell pellet was fixed by drop-wise addition of cold 70% ethanol while vortexing gently, and stored at 4°C. The pellet was washed twice and resuspended in PBS and stained with 1 μg/ml propidium iodide. Propidium iodide fluorescence was measured in 30,000 cells per sample using a Becton-Dickinson (Franklin Lakes, NJ) Facscalibur flow cytometer. The percentage of cells in each stage of the cell cycle, as well as the percentage of cells that were apoptotic (sub-G_0) was determined by analyzing the data with ModFit software. The experiment was repeated three times.

Detection of Caspase-3 Cleavage

Neuroblastoma cells were grown to 80% confluence and treated with DMSO or NDGA for 6 h. Alternatively, cells were serum starved for 4 h and cultured with or without 60 μM NDGA and with or without 10 nM IGF-I for 3 h. Lysates were collected as described above, and the 14/17 kDa cleavage fragments of caspase-3 were detected via SDS–PAGE followed by immunoblotting with anti-cleaved caspase-3 antibody.

Measurement of Cell Motility

Neuroblastoma cells were plated on gold particle-coated coverslips (prepared as described by Albrecht-Buehler [1977]) in serum-free media at a density of 25,000 cells per coverslip. The cells were incubated for 2 h to allow adhesion to the coverslip. Then, wells were treated with DMSO or 30 μM NDGA for 1 h. 1 nM IGF-I was then added to some wells. Incubation continued for 6 h, followed by fixation with 3.5% glutaraldehyde. Coverslips were mounted on glass slides, then viewed on a Lietz Orthoplan inverted microscope attached to a Sony videoprocessor. Digital images of the tracks etched into the gold by the cells from three separately treated coverslips per condition were collected at 200× magnification using Adobe Photoshop software. For each condition, the areas of 120 tracks made by individual cells were measured with NIH Image 1.61 software.

Treatment of Xenografted Nude Mice With NDGA

Nude mice xenografted with human Kelly neuroblastoma cells were treated with NDGA to determine if NDGA can affect tumor growth in vivo. Briefly, 7×10^6 Kelly human neuroblastoma cells were resuspended in a 1:1 mixture of PBS and Matrigel (BD Clontech, Inc.) and 100 μl of the mixture was injected subcutaneously into the flanks of 6- to 12-week-old BALB/c nude mice. After 1 cm tumors were established (~10–14 days post implantation), animals were injected subcutaneously with either vehicle (50% ethanol/50% Tween-80) or NDGA, (50 mg/kg, suspended in vehicle) daily for 10 days. Tumors were then harvested, weighed and measured. The formula width^2 × length/2 was used to calculate tumor volumes.

RESULTS

The Specific IGF-IR Inhibitor NVP-AEW541 Inhibits Neuroblastoma Growth In Vitro

NVP-AEW541 is a compound that specifically inhibits IGF-IR phosphorylation at low doses, and effectively inhibits the growth of several types of cancer in vitro [Garcia-Echeverria et al., 2004]. We treated SH-SY5Y (Fig. 1A) and Kelly (data not shown) neuroblastoma cells cultured on 96 well plates with NVP-AEW541 for 72 h at concentrations ranging from 0.5 to 4× the IC50 for the IGF-IR (0.086 μM), in serum containing medium, or serum-free medium supplemented with 10 nM IGF-I. Cell growth was assessed by using CyQUANT dye to measure total DNA content of the cultures (Materials and Methods). Similar to results reported by Tanno et al. [2006], NVP-AEW541 inhibited neuroblastoma growth in a dose-dependent manner.

IGF-I Stimulated IGF-IR Phosphorylation and Insulin-Stimulated InsR Phosphorylation in Neuroblastoma Cells are Inhibited by NDGA

NDGA was previously shown to inhibit the phosphorylation of the IGF-IR in partially purified preparations of the receptor and in breast cancer cell lines [Youngren et al., 2005]. Given that the insulin receptor (InsR) is structurally similar to the IGF-IR, NDGA could potentially inhibit the InsR as well. We tested the ability of NDGA to inhibit IGF-IR and InsR phosphorylation in neuroblastoma cells. Serum-starved SHEP and SH-SY5Y neuroblastoma cells were treated with either DMSO (vehicle control) or 60 μM NDGA for 1 h. The cultures were then treated for 10 min with or without 1 nM IGF-I or 10 nM insulin. Protein

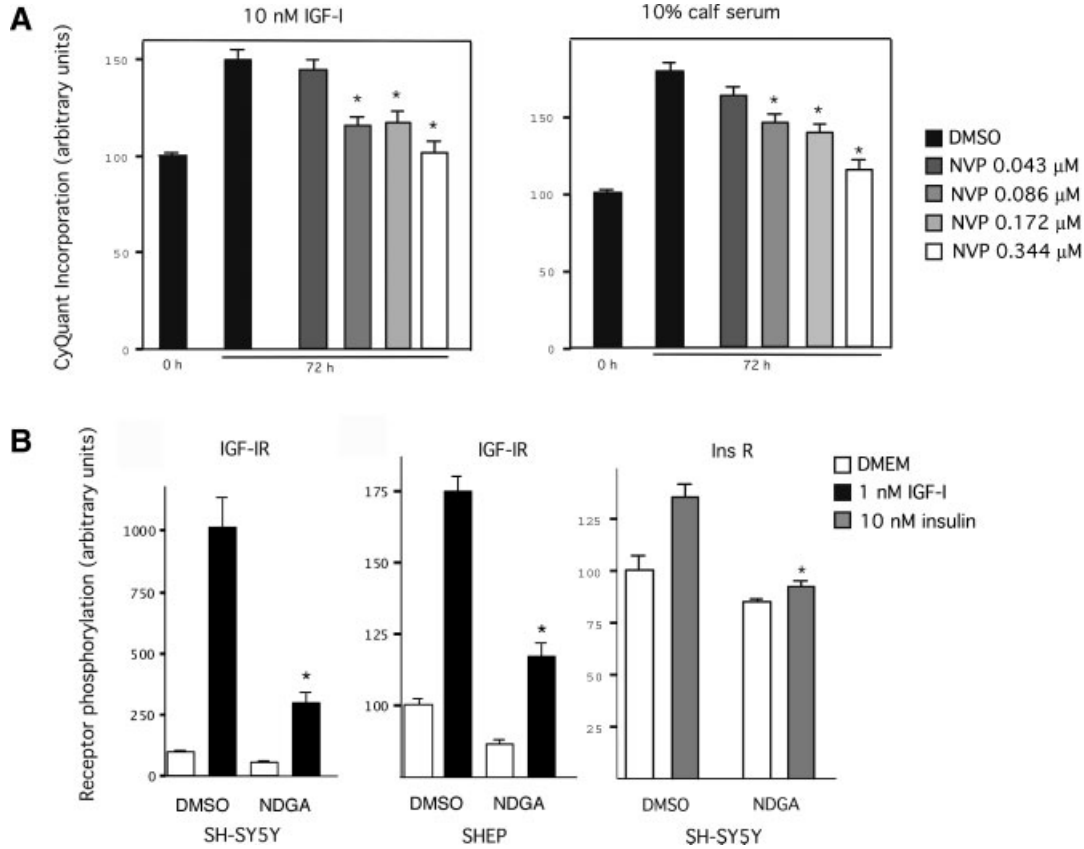

Fig. 1. **A**: NVP-AEW541 inhibits neuroblastoma growth. SH-SY5Y cells were cultured in serum-free medium supplemented with 10 nM IGF-I (left) or medium with 10% calf serum (right). Cultures were treated for 72 h with DMSO or concentrations of NVP-AEW541 representing 0.5, 1, 2, and 4 times the IC50 for the IGF-IR (0.086 μM). Culture plates were frozen at 0 h (control) or 72 h and total DNA content was measured using CyQUANT dye (Materials and Methods). Bars represent mean CyQUANT absorbance (expressed as a percentage of mean absorbance at 0 h) of three experiments (six samples per experiment) ±SEM. $^*P < 0.05$ compared to DMSO treatment at 72 h. **B**: NDGA inhibits IGF-IR and InsR phosphorylation in neuroblastoma cells. SH-SY5Y and SHEP neuroblastoma cells were serum starved and treated with DMSO or 60 μM NDGA for 1 h. Then, 1 nM IGF-I or 10 nM insulin was added to some the cultures for 10 min. IGF-IR and InsR phosphorylation were detected by ELISA (Materials and Methods). Results are mean ± SEM for measurements collected in all experiments expressed relative to receptor phosphorylation in unstimulated DMSO-treated cells. The experiment was repeated three times, with three replicates of each condition performed in each experiment. $^*P < 0.01$ versus DMSO + IGF-I or insulin.

lysates were collected and the degree of IGF-IR or InsR phosphorylation in the lysate samples was quantified using ELISA (Fig. 1B). IGF-I induced an increase in IGF-IR tyrosine phosphorylation in both cell lines, which was inhibited by NDGA. Insulin-stimulated InsR phosphorylation was also potently inhibited by NDGA in SH-SY5Y cells.

NDGA Inhibits Neuroblastoma Proliferation

Since both NVP-AEW541 and NDGA inhibit IGF-IR activation, we looked to see if NDGA could inhibit neuroblastoma growth, similar to NVP-AEW541. SH-SY5Y and Kelly neuroblastoma cells were cultured on four 96-well plates in serum-free media supplemented with 10 nM IGF-I for up to 72 h. At 0 h, the cultures were treated with 15–120 μM NDGA, or DMSO as control. CyQUANT staining was again used to measure the total DNA content in the cultures. NDGA inhibited proliferation of SH-SY5Y and Kelly cells, and caused cell death at higher doses (Fig. 2).

To determine if NDGA would still have an inhibitory effect on neuroblastoma growth in serum, where other factors could contribute to neuroblastoma mitogenesis and survival in addition to IGFs, the experiment was repeated using SH-SY5Y, SHEP, and Kelly cells cultured in medium containing 10% calf serum (Fig. 3). NDGA inhibited neuroblastoma proliferation in serum up to 72 h. Higher doses of NDGA again caused cell death. These results demonstrate that NDGA inhibits the growth and survival of

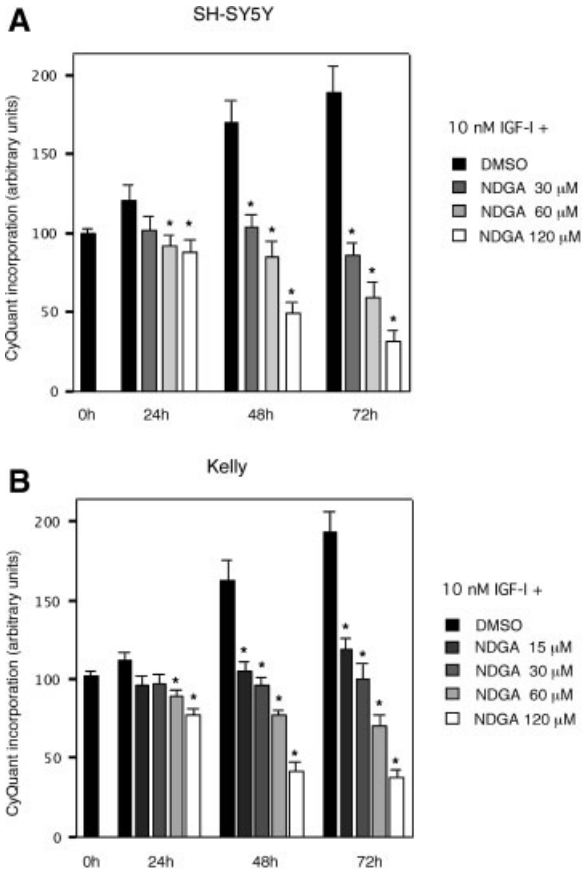

Fig. 2. NDGA inhibits neuroblastoma growth in serum-free medium supplemented with 10 nM IGF-I. SH-SY5Y (**A**) and Kelly (**B**) neuroblastoma cells were cultured in serum-free medium with 10 nM IGF-I and treated with DMSO or NDGA (15–120 μM). DNA content was measured at 0, 24, 48, and 72 h using CyQUANT staining (Materials and Methods). Means ± SEM from three separate experiments are expressed as a percentage of absorbance at 0 h. $^*P < 0.05$ versus DMSO-treated control at the same time point.

neuroblastoma cells supported by either serum or IGF-I.

The Growth Inhibition and Death Promoting Effects of NDGA are not Attributable to Lipoxygenase Inhibition

NDGA was originally identified as an inhibitor of 5-, 12-, and 15-lipoxygenases with IC50s similar or lower to its IC50 for the IGF-IR [Youngren et al., 2005]. Therefore, lipoxygenases are likely to be inhibited at the dose ranges used in the previous experiments. We used three specific lipoxygenase inhibitors unrelated to NDGA to see if inhibition of lipoxygenases could suppress neuroblastoma growth and survival. The lipoxygenase inhibitor CDC has an IC50 for 12-lipoxygenase of 0.063 μM, while caffeic acid inhibits 5- and 15-lipoxygenases (IC50s of 3.7 μM and 2.2 μM, respectively); using them in combination can effectively inhibit lipoxygenases at low drug doses. ETI was also used separately, as it inhibits all three lipoxygenases at an IC50 similar to that for NDGA (3 μM for 5-, and 20 μM for both 12- and 15-lipoxygenases). SH-SY5Y and Kelly cells cultured on 96-well plates were treated with DMSO, NDGA, a combination of CDC and caffeic acid, or ETI, at doses of at least 3× the IC50s for their respective lipoxygenases for 72 h, and growth was assessed with CyQUANT. Neither ETI nor the combination of CDC and caffeic acid affected SH-SY5Y cells grown in serum (Fig. 4A, left). The growth of Kelly cells was slightly inhibited by the lipoxygenase inhibitors, but this inhibition was minimal compared to the effect of NDGA (Fig. 4A, right).

NDGA Prevents IGF-I Activation of the MAPK Pathway

Neuroblastoma mitogenesis is regulated by IGFs via activation of the MAPK signaling pathway [Kim et al., 1997], leading to the phosphorylation and activation of ERK 1 and 2. If NDGA inhibits IGF-IR activation, it would likely prevent IGF-I-induced ERK phosphorylation. Serum starved SH-SY5Y and SHEP cells were treated for 1 h with DMSO or increasing concentrations of NDGA, and then stimulated with 10 nM IGF-I for 15 min. Lysates were collected and proteins separated by SDS–PAGE as described in Materials and Methods. ERK phosphorylation was assessed by immunoblotting with anti-phospho ERK1/2 antibody. ERK phosphorylation was increased by IGF-I in SH-SY5Y cells (Fig. 4B, upper). NDGA inhibited IGF-stimulated phosphorylation of ERK in a dose-dependent manner. Similar results were obtained in SHEP cells (data not shown).

Akt Phosphorylation is Inhibited by NDGA

IGFs promote neuroblastoma survival by activation of the PI-3K-dependent activation of Akt [Van Golen and Feldman, 2000; Van Golen et al., 2000]. The effect of NDGA on IGF-stimulated Akt activation was assessed in serum-starved SH-SY5Y and SHEP cells, via SDS–PAGE and Western immunoblotting as described above. Similar to the effects on ERK phosphorylation, NDGA caused a dose-dependent inhibition of IGF-stimulated

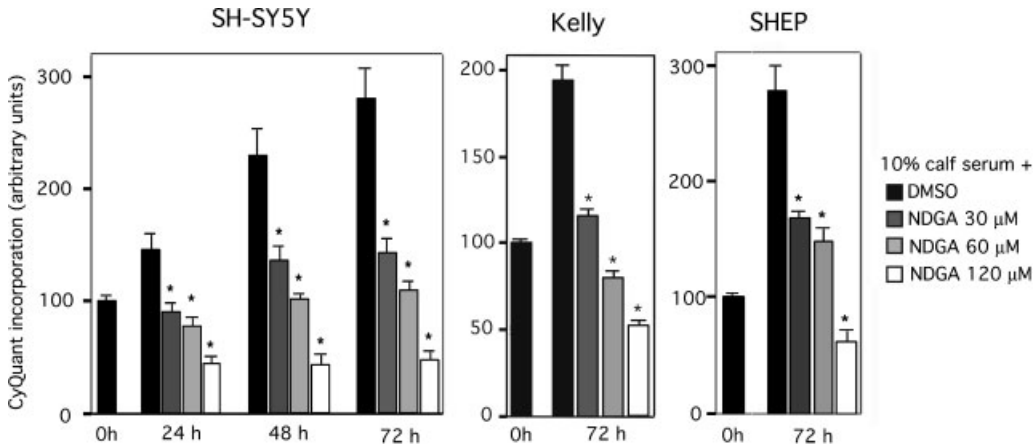

Fig. 3. NDGA inhibits neuroblastoma growth in serum. SH-SY5Y, SHEP, and Kelly neuroblastoma cells were cultured in serum and treated with DMSO or NDGA (30–120 μM). Samples were collected and processed for CyQUANT absorbance as in Figure 2. Only the 0 h and 72 h time points are shown for SHEP and Kelly cells. Each bar represents the mean ± SEM of three separate experiments, and with six replicates of each condition per experiment. $*P < 0.05$ versus DMSO-treated control at the same time point.

Akt phosphorylation in SH-SY5Y and SHEP cells (Fig. 4B, lower, and data not shown).

Caspase-3 is Activated by NDGA

Akt activation supports neuroblastoma survival by suppressing apoptosis, in part by preventing the catalytic activation of caspase-3 [Van Golen and Feldman, 2000; Van Golen et al., 2000]. Disruption of Akt signaling could increase activation of caspase-3 and thus drive neuroblastoma cells into apoptosis. To determine if NDGA causes caspase-3 activation, SH-SY5Y neuroblastoma cells were cultured in serum and treated with NDGA for 6 h. Caspase-3 activation was assessed by SDS–PAGE and immunoblotting with anti-cleaved caspase-3 antibody, which detects the small cleavage fragments of caspase-3 that are released upon its activation. NDGA caused dose-dependent caspase-3 activation (Fig. 5A). To determine if exogenous IGF-I was able to prevent this activation, SH-SY5Y cells were cultured in serum-free media containing 10 nM IGF-I and simultaneously treated with NDGA or DMSO as a control. Caspase-3 activation was still detectable when NDGA-treated cells were given IGF-I (10 nM; Fig. 5B). Caspase-3 activation was not detected in serum-starved cells cultured in the absence of IGF-I, while NDGA treated SH-SY5Y cells cultured in the absence of IGF-I, which secrete their own IGF-II, showed strong caspase activation. This suggests NDGA is capable of both pushing the cells towards apoptosis and suppressing IGF-mediated rescue.

NDGA Causes Neuroblastoma Cells to Undergo Apoptosis

SH-SY5Y cells cultured in serum were treated with DMSO or NDGA (30–120 μM). After 24 h, the cells were harvested and subjected to flow cytometric cell cycle analysis as described in Materials and Methods. NDGA caused a dose-dependent increase in the percentage of sub-G_0 cells, the fraction of cells undergoing apoptosis (Fig. 5C).

IGF-Stimulated Cell Motility is Inhibited by NDGA

IGFs increase the motility of neuroblastoma cells, in part through PI-3K signaling [Meyer et al., 2001, 2005]. The ability of NDGA to impact neuroblastoma motility was assessed by measuring the motility of serum starved SHEP and SH-SY5Y cells treated with or without 1 nM IGF-I (the optimal dose for stimulating motility, Meyer et al., 2001). Motility was quantified by plating the cells on coverslips coated with fine gold particles, and then measuring the areas cleared of particles by the cells while they moved during a 6 h incubation. IGF-I increased the motility of SH-SY5Y and SHEP cells, and 30 μM NDGA strongly suppressed this increase in motility (Fig. 6A).

NDGA Inhibits Tumor Growth in a Xenograft Model of Neuroblastoma

To determine if NDGA's anti-tumor effects in vitro could be duplicated in vivo, nude mice

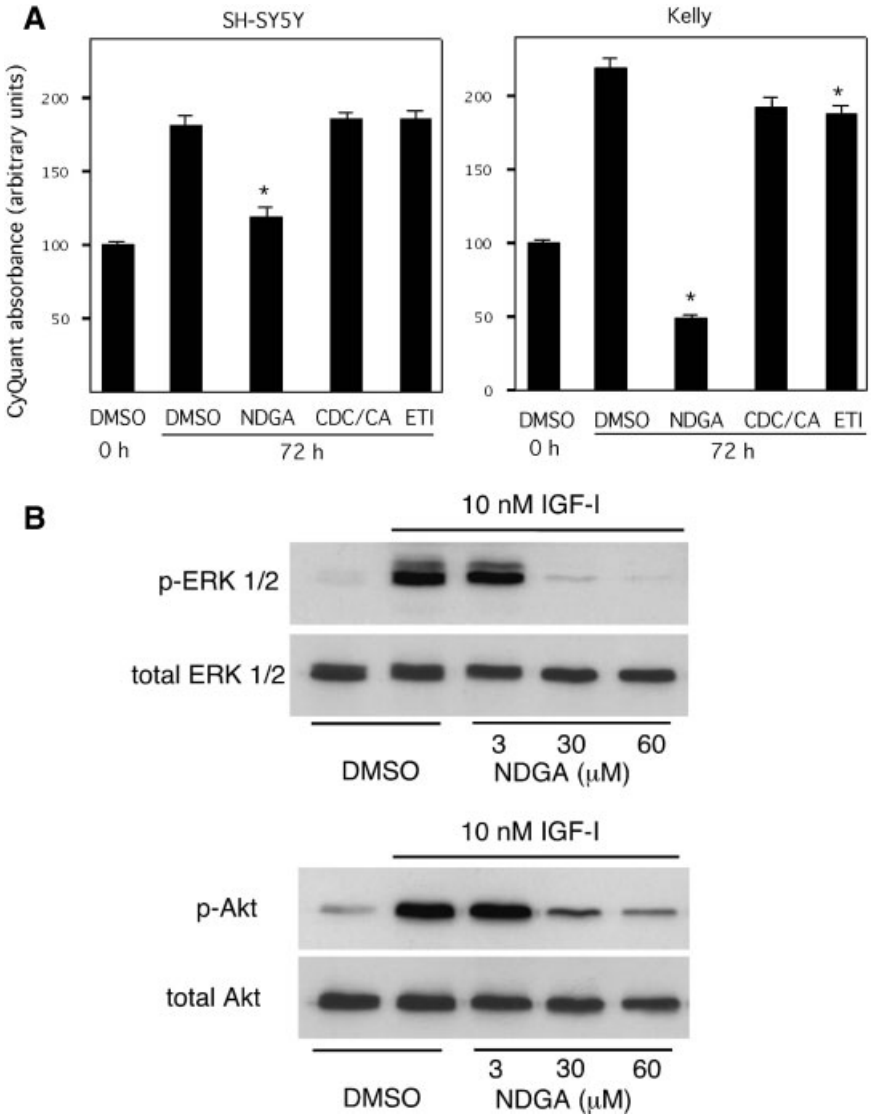

Fig. 4. **A**: Inhibition of lipoxygenases does not account for the inhibitory affect of NDGA on neuroblastoma growth. SH-SY5Y (left) and Kelly (right) cell were cultured in serum-containing medium, and treated for 72 h with DMSO, 90 μM NDGA, a combination of CDC and caffeic acid (CDC/CA) or ETI. The concentrations of CDC/CA and ETI were equivalent to 90 μM NDGA in terms of lipoxygenase inhibition; that is, all drugs were used at ~3× the IC50 for lipoxygenases (see Materials and Methods). Bars represent mean CyQUANT absorbance expressed as a percentage of mean absorbance at 0 h, ±SEM. $^*P < 0.05$ compared to DMSO treatment at 72 h. **B**: IGF-I-stimulated ERK and Akt activation are blocked by NDGA. SH-SY5Y cells were serum starved and treated with DMSO or 3, 30, or 60 μM NDGA for 1 h, then given 10 nM IGF-I for 15 min. Lysates were collected and ERK (**upper panel**) or Akt (**lower panel**) phosphorylation was detected via Western blot analysis. Total ERK and total Akt are shown to confirm equal loading of protein in all lanes. Representatives of three separate experiments are shown.

with established Kelly cell xenografts were treated with NDGA (50 mg/kg i.p. daily) or vehicle. After 10 days of treatment, all mice were sacrificed because the tumors in the vehicle treated mice had grown larger than is permitted by our institutional animal care rules. The NDGA treated animals, however, showed 50% less tumor growth (Fig. 6B).

DISCUSSION

The IGF signaling system has become a target of increasing interest in cancer therapy research. A variety of approaches to disrupting this system have been investigated, including use of anti-receptor antibodies, anti-sense nucleotides, ligand mimicking compounds,

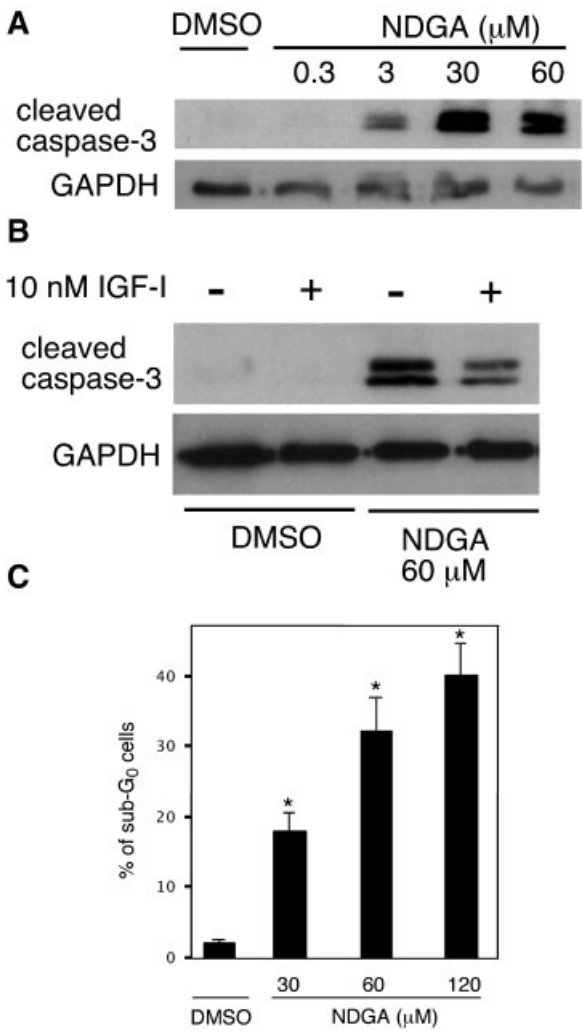

Fig. 5. NDGA causes caspase activation and apoptosis in neuroblastoma cells. **A**: SH-SY5Y cells grown in serum containing-medium were treated with DMSO or 0.3–60 μM NDGA for 12 h. Activated caspase-3 fragments were detected using Western blot analysis. **Upper panel** shows the 14/17 kDa cleavage fragments of caspase-3, while the **lower panel** shows GAPDH expression as a loading control. A representative of three separate experiments is shown. **B**: Serum-starved SH-SY5Y cells were treated with DMSO or 60 μM NDGA for 12 h. Some cultures included 10 nM IGF-I for the entire treatment period. Lysates were collected and caspase-3 cleavage fragments were detected as above. **C**: SH-SY5Y cells were treated with DMSO or NDGA (30–120 μM) for 24 h, fixed, stained with propidium idodide, and subjected to flow cytometric analysis of cell cycle. Bars represent the mean ± SEM percentage of cells in the sub-G_0 apoptotic phase from five separate experiments. *$P < 0.05$ versus DMSO.

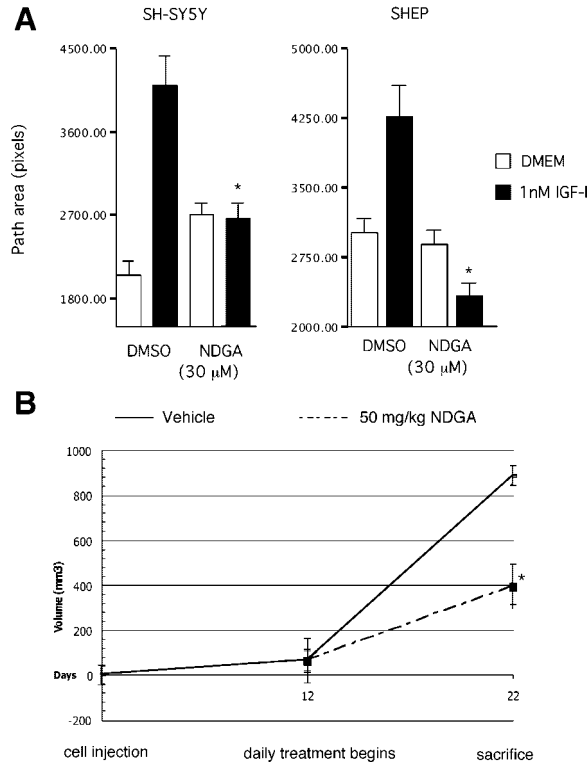

Fig. 6. NDGA inhibits IGF-I stimulated motility and in vivo neuroblastoma tumor growth. **A**: SH-SY5Y and SHEP cells were plated on gold particle-coated coverslips in serum-free conditions. After adhering, the cells were treated with DMSO or 30 μM NDGA for 1 h. Half the cultures were then treated with 1 nM IGF-I, and incubation continued for 6 h. The track areas of cells that were etched into the gold particle coating were measured using NIH Image software. Each bar represents the mean ± SEM of 120 individual track areas collected from three separate experiments. *$P < 0.001$ versus DMSO + 1 nM IGF-I. **B**: Kelly neuroblastoma cells were implanted subcutaneously in nude mice as described in Materials and Methods. When palpable tumors formed (day 12), mice were treated with daily i.p. injections of 50% ethanol/50% Tween-80 (vehicle, solid line) or 50 mg/kg NDGA (dashed line). On day 22, the animals were sacrificed and tumor volume was measured with calipers. Error bars are SD. N = 4 animals in each treatment group. *$P < 0.002$ versus vehicle control.

IGF-binding proteins, and small molecule inhibitors [Dake et al., 2004; Foulstone et al., 2005]. A related pair of relatively specific and potent inhibitors of the IGF-IR, NVP-ADW742, and NVP-AEW541, inhibit the growth of a variety of tumors in vitro and in vivo, including fibrosarcoma and neuroblastoma [Garcia-Echeverria et al., 2004; Mitsiades et al., 2004; Tanno et al., 2006]. Still, relatively few agents have been identified that have effects against the IGF-IR, and clinical studies for the highly specific NVP compounds are not anticipated. Considering the promising pre-clinical results of anti-IGF treatment in numerous malignancies, more candidate agents need to be characterized to increase the chances of finding clinically viable options that combine efficacy and low toxicity.

NDGA has had a long history of use as a lipoxygenase inhibitor before it was recently found to inhibit the tyrosine phosphorylation of the IGF-IR [Youngren et al., 2005]. NDGA has been tested as a potential anti-cancer agent in several studies, where it induced apoptosis and suppressed mitogenesis [Vondracek et al., 2001; Seufferlein et al., 2002; Tong et al., 2002; Hoferova et al., 2003]. Some of these studies hypothesized that suppressing lipoxygenase-dependent prostaglandin synthesis may mediate inhibition of tumor growth. We find in this study that inhibition of lipoxygenases with specific inhibitors unrelated to NDGA has no appreciable effect on neuroblastoma growth. Rather, we propose that the anti-tumor effect of NDGA in neuroblastoma cells is at least partly mediated through inhibition of the IGF-IR.

Neuroblastoma cells are highly dependent upon paracrine and autocrine IGFs for growth [Martin and Feldman, 1993; Meghani et al., 1993; Leventhal et al., 1995; Kiess et al., 1997], and thus it is logical that agents like NVP-AEW541 and NDGA that are capable of blocking IGF-IR activation would inhibit neuroblastoma tumorigenesis. Neuroblastoma cell lines that secrete IGF-II are capable of serum-independent growth [Martin and Feldman, 1993; Leventhal et al., 1995] and cell lines that express high levels of the IGF-IR are more aggressively tumorigenic [Singleton et al., 1996b]. We find that NDGA at low doses (15–30 μM) completely blocks neuroblastoma growth over a period of several days in vitro, both in serum, and in serum-free conditions where added and autocrine IGFs support neuroblastoma growth. NDGA prevents IGF-I-mediated activation of both the IGF-IR and ERK 1 and 2 in neuroblastoma cells at the same doses that inhibit growth in vitro. The growth of Kelly neuroblastoma tumor xenografts in nude mice is also suppressed by NDGA. Additional studies with NDGA in xenografted animals are in preparation to further characterize its efficacy, impact on survival, and ability to inhibit putative target signaling pathways in vivo. In addition to neuroblastoma, NDGA inhibits the in vitro and in vivo proliferation of other cancers that are highly responsive to IGFs, including lung [Moody et al., 1998] and breast [Youngren et al., 2005].

IGFs are also potent stimulators of neuroblastoma survival, causing strong activation of Akt while suppressing caspase-3 activation [Van Golen and Feldman, 2000; Van Golen et al., 2000]. In neuroblastoma, NDGA causes inhibition of IGF-stimulated Akt activation and is strongly apoptotic, causing caspase-3 activation and a large increase in sub-G_0 cells. Similar results are seen in breast cancer cell lines treated with NDGA, where Akt activation is suppressed and BAD activation is increased [Youngren et al., 2005]. It is possible that disruption of other unknown targets of NDGA could lead to apoptosis in neuroblastoma cells. However, 10 nM IGF-I is known to completely prevent caspase-3 activation when the stressor that induces apoptosis is unrelated to IGF signaling (e.g., osmotic stress [Van Golen and Feldman, 2000; Van Golen et al., 2000]). In our experiments caspase-3 activation remained strong following administration of 10 nM IGF-I, suggesting that IGF-stimulated rescue is impaired by NDGA treatment.

IGF-I stimulates neuroblastoma cells to undergo organized actin polymerization and lamellipodium extension, resulting in increased cell motility [Kim and Feldman, 1998; Meyer et al., 2001, 2005]. Increased cell motility, along with the ability to digest extracellular matrix, affords cancer cells greater ability to invade tissues and blood vessels, leading to metastasis and diffuse tissue dissemination. This is of particular concern with neuroblastoma, where tumor invasion of bone, a site of high IGF production, is associated with poor response to therapy. We find that NDGA effectively inhibits IGF-I stimulated motility of neuroblastoma.

NDGA does not show high selectivity for a single receptor, in contrast to NVP-ADW742 and NVP-AEW541, and should not be viewed as solely an IGF-IR inhibitor. NDGA likely works on a subset of receptor tyrosine kinases, including the IGF-IR, InsR, and her2/neu receptor [Youngren et al., 2005]. NDGA inhibits the activation of the PDGF receptor and PDGF-stimulated DNA synthesis [Domin et al., 1994]. However, Seufferlein et al. [2002], found no affect of NDGA on EGF receptor phosphorylation. More work is needed to characterize which additional receptors may be affected by NDGA treatment.

As the IGF-I and insulin receptors are highly homologous, part of the effect of NDGA against neuroblastoma tumorigenesis might be mediated through InsR inhibition. However, insulin is 200 times less potent than IGF-I at stimulating SH-SY5Y proliferation, and at least

one-third of insulin's effect on proliferation is mediated by activation of the IGF-IR, not InsR [Meghani et al., 1993]. Moreover, plasma concentrations of IGF-I are 100–1,000 times more concentrated than insulin (nanomolar for IGF-I, picomolar for insulin). Thus, inhibition of InsR activity is unlikely to account for significant anti-tumor effects of NDGA in neuroblastoma.

Of note, the anti-InsR activity of NDGA could be predicted to cause a diabetic phenotype. Paradoxically, NDGA has an anti-diabetic effect on rats, decreasing serum glucose and triglycerides without affecting insulin levels [Luo et al., 1998; Gowri et al., 1999; Scribner et al., 2000]. NDGA was previously considered for treatment of diabetes because of its inhibition of prostaglandin synthesis. Thus, NDGA's inhibition of insulin receptors may not result in a diabetes-like toxicity because of its concomitant effects on prostaglandin synthesis. NDGA analogs are being developed in an attempt to achieve better specificity (J. Youngren and I. Goldfine, unpublished work), and some have been tested for efficacy against lung cancer [Moody et al., 1998]. Further characterization of these analogs may lead to the discovery of agents more specific for individual receptor tyrosine kinases.

An important potential advantage held by NDGA over other available agents with high IGF-IR specificity is its apparent safety in humans. A phase I trial of orally administered NDGA in prostate cancer patients at the University of California, San Francisco, is nearing completion with no observed dose limiting toxicities (personal communication, Charles Ryan, UCSF Urology). Thus, it is anticipated NDGA will soon be available for study in children, and its anti-IGF signaling and anti-tumorigenic effects in neuroblastoma warrant study of this agent's potential for the treatment of neuroblastoma.

In summary, NDGA effectively suppresses neuroblastoma growth in vitro and in vivo, and inhibits the motility and promotes the apoptosis of neuroblastoma cells in culture. These effects appear to be mediated, at least in part, through inhibition of IGF-IR signaling. Future studies will investigate whether NDGA might be even more effective in combination with treatments that affect other aspects of neuroblastoma tumorigenesis, such as anti-myc agents or radiation. NDGA could also be used in combination with other agents that target the IGF-IR, modulate ligand-receptor interactions, or that target downstream components of the IGF signaling pathway, such as IGF binding proteins or anti-PI-3K agents.

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