Nordihydroguaiaretic acid restores expression of silenced E-cadherin gene in human breast cancer cell lines and xenografts

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In our study we use nordihydroguaiaretic acid (NDGA), the naturally occurring lignan, to investigate whether it plays a role in the prevention and treatment of cancer by epigenetic modifications. The growth inhibitory effect of NDGA on human breast cancer cell lines was determined using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay). It substantially inhibited the growth of human breast cancer cell lines SKBR3 and MDA-MB-435 with an estimated IC_{50} of 31.09 ± 1.6 and 38.8 ± 2.1 μmol/l respectively, after 4 days incubation with different NDGA concentrations. The in-vivo anticancer activity of NDGA was evaluated by calculating the tumor growth inhibition value. NDGA substantially inhibited the growth of human breast carcinoma cells in both animal and cell-based models. We also found that a single treatment with NDGA reactivates methylation-silenced E-cadherin gene *in vitro* and *in vivo*, suggesting an intriguing concept that lignans may act as natural effective epigenetic modifiers in the prevention and treatment of cancer. *Anti-Cancer Drugs* **19:487–494 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Naturally occurring plant phytoestrogens have recently received a great deal of attention for the potential health benefits they may provide. Recently, interest has been focused on the cancer-protective role of several phytoestrogens such as lignans and isoflavonoids. Although isoflavones have dominated phytoestrogen cancer research, lignans may be more relevant in Western countries especially in North American diets. The purified lignans or lignan-rich diets have been implicated as a possible link to the etiology, promotion, and progression of breast cancer risk modulation [1]. Experimental evidence has shown anticarcinogenic activities, including antiestrogenic, antioxidant, and anti-inflammatory effects of lignans [2]. Nordihydroguaiaretic acid (NDGA), a lignan found in high amounts in *Larrea tridentate*, has estrogenic activity *in vitro* and *in vivo* [3,4]. Several studies on the effects of NDGA on various cancer cell lines have shown promising results [5,6]. Still, the mechanism of its anticancer effect is not well understood. Recent work from our laboratory has demonstrated that NDGA may cause demethylation of p16, restore its expression and function in human breast T47D cancer cell lines. Here we evaluated another two breast carcinoma cell lines SKBR3, MDA-MB-435, and an MDA-MB-435 mouse xenograft model, which have a densely methylated E-cadherin promoter [7]. The results show that NDGA is able to demethylate and induce expression of tumor suppressor genes known to be silenced *in vitro* and *in vivo*.

Tumor invasion with subsequent metastases is the major cause of morbidity and mortality in patients with breast cancer. Data from various studies suggest that breast E-cadherin is expressed in normal adults in luminal epithelial cells, and it exerts a potent invasion suppressing role in tumor cell lines and in in-vivo tumor model systems [8,9]. As an important invasion tumor suppressor, the aberrant expression of E-cadherin has been associated with the development of metastases in patients with breast cancer. Hypermethylation of the E-cadherin promoter and the overlapping 5′CpG island has been demonstrated to correlate with loss of E-cadherin expression at the transcriptional level for various breast cancer cell lines and primary ductal breast cancers. Several infiltrative lobular cancers were recently reported to carry methylated E-cadherin promoter sequences [10]. A large body of experimental evidence has demonstrated that reexpression of silenced tumor suppressor genes by demethylating drugs leads to a strong inhibitory effect on

cancer cell growth *in vitro* and *in vivo* [11]. Treatment of two breast cancer cell lines Hs578t and MDA-MB-231 with the DNA methylation inhibitor 5-aza-2-deoxycytidine resulted in slight upregulation of E-cadherin mRNA and protein levels [12]. Thus reversing gene-promoter methylation of tumor suppressor genes, an attractive target for anticancer treatment.

Materials and methods

Chemical agents

NDGA was purchased from Sigma (St Louis, Missouri, USA). For all in-vitro applications, NDGA was dissolved in absolute dimethylsulfoxide (DMSO, Sigma) as 100 μmol/l and 1 mmol/l stock solution and stored at −20°C. NDGA was dissolved in 6% Cremophor EL, 6% ethanol, and 88% saline (volume/volume/volume) for in-vivo applications. Its solution was prepared fresh for each experiment.

Cell lines

Human breast cancer cell lines SKBR3 and MDA-MB-435 were obtained from Peking Union Medical College and Chinese Academy of Medical Sciences. Cells were grown in RPMI1640 and L-15 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. The medium was changed daily. Cells were dislodged for both passaging and harvesting by a brief incubation in 0.25% trypsin and 0.02% EDTA. Cells were stained with trypan blue and counted using a hematocytometer.

Cells were treated for 7 days with various concentrations of NDGA (0, 10, 20, 50, and 100 μmol/l), whereas only DMSO (solvent) was added for the control regimen.

DNA and RNA extraction

Total genomic DNA was isolated using phenol : chloroform : isoamyl alcohol (25 : 24 : 1) according to standard protocol and precipitated with 2.5 volumes ethanol. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions.

MTT cell proliferation assay

Cell proliferation was measured using the MTT assay. Approximately 5×10^3 cells were seeded in each well of a 96-well plate. After 24 h adherence to the plate, the cells were treated with serial dilutions of NDGA for 4 days. At the end of incubation, 10 μl of 5 mg/ml MTT (Sigma) was added to each well. After 4 h incubation, the medium was aspirated and 150 μl DMSO was added to each well. The plates were mixed gently by rocking back and forth until the blue sedimentation was completely dissolved. The plates were analyzed by an enzyme-linked immunosorbent assay reader at a wavelength of 570 nm. The growth inhibition rates were calculated using the following formula: percentage inhibition = (1 − average optical density value of experimental wells/average optical density value of control wells) × 100%.

Bisulfite modification-based cytosine methylation analysis of E-cadherin

The conversion of DNA by sodium bisulfite was performed using an established protocol with modifications [13]. Initially, 1–2 μg of genomic DNA was denatured with 3 mol/l NaOH, followed by incubation with freshly prepared 2.5 mol/l sodium bisulfite/1 mol/l hydroquinone, pH 5.0, in a total volume of 400 μl, at 50°C for 16 h. DNA was purified with the Wizard DNA Clean-UP System (Promega, Madison, Wisconsin, USA). Modification of the DNA was completed by the addition of 10 μl of NaOH 3 mol/l at room temperature during 15 min. Precipitation was carried out by the addition of 166 μl of ammonium acetate 5 mol/l (pH 7.0) and 800 μl of ethanol. The bisulfite-modified DNA was resuspended in 20 μl of sterile water and stored at −20°C.

The methylation pattern within the CpG island of the E-cadherin gene was determined using methylation-specific PCR (MSP) following a previously published method [14]. Bisulfite-treated DNA was amplified using the primers M-forward (5′-GGTGAATTTTTAGTTAATT AGCGGTAC-3′), M-reverse (5′-TAACTAAAAATTCACC TACCGAC-3′), U-forward (5′-TAATTTTAGGTTAGAG GGTTATTGT-3′), and U-reverse (5′-CACAACCAATC AACAACACA-3′) [15,16]. HotStarTaq DNA polymerase (QIAGEN GmbH, Hilden, Germany) was used for amplification. The product sizes of the methylated and unmethylated amplicons were 204 and 97 bp, respectively. Water blanks were included in each assay. The PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide.

The resulting PCR products were fractionated in low melting agarose (Sigma), purified using Wizard SV Gel and the PCR Clean-Up System kit (Promega). The purified PCR products were cloned into the pGEM-T Easy vector (Promega) and automated sequencing was carried out using the DNA Sequencer ABI Prism 3730 Avant genetic analyzer (Applied Biosystems, Foster city, California, USA).

RT-PCR

Total RNA was isolated from cultured cells using Trizol (Invitrogen) and first-strand complementary DNA (cDNA) synthesized from 2 to 5 μg total RNA using SuperScript II reverse transcriptase (Invitrogen) as directed by the manufacturer. The synthesized cDNA was diluted to 100 μl with ultrapure water. RT-PCR product was generated by using E-cadherin-specific sense (5′-GTCTACAAAAGGACAGCTAT-3′) and antisense (5′-TTGTTGGATTTGATCTCAACC-3′) primers. Primers specific for the GAPDH cDNA were used to

confirm the cDNA integrity, and oligonucleotide primers were designed to amplify the E-cadherin cDNAs. Negative controls for the PCR were run under the same conditions without reverse transcriptase. The PCR products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized by ultraviolet light. A semiquantitative determination of the level of E-cadherin expression was made by comparing the densities of the 361-bp E-cadherin and the 226-bp GAPDH products.

Western blot analysis

Cells were pelleted and lysed in ice-cold lysis buffer (CelLytic M Cell lysis reagent, Sigma), then centrifuged at 12 000*g* for 20 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay, (BCA) kit (Pierce, Rockford, Illinois, USA). Samples were boiled for 5 min and loaded onto 8% acrylamide gel (100 μg/lane). Predetermined molecular weight standards (Invitrogen) were used as markers. Protein on the gel was blotted onto nitrocellulose membranes at 225 mA for 120 min. After transfer, the membranes were incubated with blocking buffer [5% nonfat milk in wash buffer (Tris-buffered saline with Tween and 0.1% Tween 20)] for 1 h at room temperature.

Protein expression of E-cadherin protein was detected by incubating with monoclonal E-cadherin antibodies HECD-1 (Takara Biomedicals, Shiga, Japan) at a dilution of 1 : 1500 overnight at 4°C. The membranes were washed three times with wash buffer and were then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) at dilutions of 1 : 2000 for 1 h at room temperature. The membrane was detected with 1 mol/l Tris–HCl (pH 9.5) containing 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine and nitro blue tetrazolium (Promega) for 5–30 min. The nitrocellulose sheets were washed with deionized water and dried with air. Blots were scanned and optical densities of the bands were quantitated.

Tumorigenesis assay in nude mice

Female, T cell-deficient, nude (nu/nu) mice, 3 weeks old were purchased from Beijing Laboratory Animal Research Center (Peking Union University, China). They were housed in a pathogen-free isolation facility with rodent chow and water *ad libitum*, and treated in accordance with institutional guidelines for animals. MDA-MB-435 cells (5×10^6 in 0.1 ml phosphate-buffered saline sterile) were injected subcutaneously (s.c.) into the flanks of nude mice. Once visible tumors were visible 7 days after injection, the animals were assigned to a treatment group and received NDGA, or to a control group that received vehicle only. Each group was composed of five mice.

Nordihydroguaiaretic acid treatment of MDA-MB-435 xenograft tumors in mice

Nude mice bearing MDA-MB-435 xenografts were treated with NDGA given intraperitoneally in a Cremophor EL-based solvent system [6% Cremophor EL, 6% ethanol, and 88% saline (volume/volume/volume)]. Mice received 100 μl containing 2 mg NDGA (100 mg/kg) or 100 μl of vehicle solvent three times per week for 7 weeks.

Evaluation of antitumor effect

Tumors were measured in two perpendicular dimensions twice a week, and the tumor volumes were calculated according to the following formula: $V(\text{cm}^3) = a^2 \times b/2$, where a is the width of the tumor (smaller diameter) and b is the length (larger diameter) [17]. The relative tumor volume (RTV) of each tumor was defined as the ratio of the volume at a given time and the volume at the start of treatment [18]. The mean RTV and standard error were calculated for each treatment group. Antitumor activity was determined by calculating the tumor growth inhibition (TGI) value using the following equation: $\text{TGI\%} = \text{T/C} \times 100$; where T is the mean RTV of the treated tumors at the experiment end point and C is the mean RTV of the control group [17,18].

The body weights of the mice were monitored for toxicity. The mice were sacrificed on day 56 after treatment. Weights of the tumors from the NDGA-treated animals were smaller than those from the vehicle-treated group. Part of the tumor tissue was immediately frozen and the remainder was fixed in 10% neutral-buffered formalin and embedded in paraffin.

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue sections of MDA-MB-435-induced tumors in nu/nu mice using the streptavidin–biotin–peroxidase method by HRP-diaminobenzidine system staining kit (R&D Systems, Minneapolis, Minnesota, USA). The primary antibodies, HECD-1 (Takara Biomedicals) were used in this study. The same protocol was carried out for negative controls, in which either the primary antibody was omitted or an isotype-matched control antibody was used. For the image, cytometric analysis was performed with Image-Pro Plus (Media Cybernetics, Inc., Bethesda, Maryland, USA).

Statistical analysis

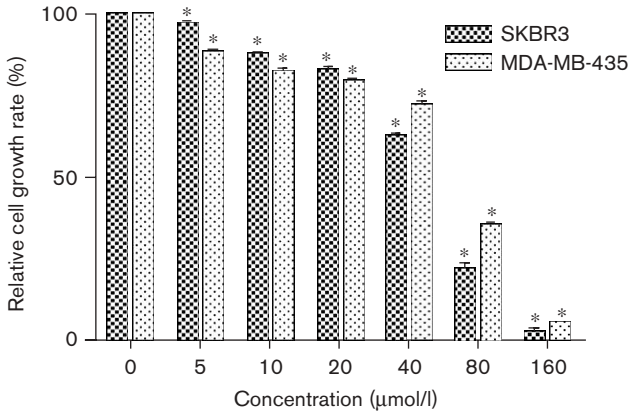
The results of all in-vitro and in-vivo assays were expressed as mean ± SD. Student's two-sided *t*-test was used to compare the values of the test and control samples. $P < 0.05$ was considered significant.

Results

Effects of nordihydroguaiaretic acid on the proliferation of SKBR3 and MDA-MB-435 cells

To determine the proliferation effects of NDGA on breast cancer cell lines, SKBR3 and MDA-MB-435 cells were treated with various doses of NDGA for 4 days and cell viability was examined using the MTT assay. A dose-dependent inhibition was seen between 5 and 160 μmol/l

Fig. 1



Effect of NDGA on cell proliferation in cultured SKBR3 and MDA-MB-435 cells. After 4 days of exposure to various concentrations of NDGA, cells were evaluated for viability. The values presented are mean ($n=3$); bars, ± SD. * Indicates statistically significant ($P<0.05$). NDGA, nordihydroguaiaretic acid.

NDGA, with the estimated IC_{50} being 31.09 ± 1.6 and 38.8 ± 2.1 μmol/l for SKBR3 and MDA-MB-435 cell lines, respectively (Fig. 1).

Reversal of hypermethylation and reactivation of E-cadherin by nordihydroguaiaretic acid in breast cancer cell lines

Previous studies have revealed that the E-cadherin gene is methylated in SKBR3 and MDA-MB-435 cell lines [7,19]. The methylation status of E-cadherin gene was examined by MSP analysis. We demonstrated that the single treatment of both cell lines with NDGA decreased the methylation status of the promoter region of the E-cadherin gene, whereas the unmethylated status of this gene was increased. After treating the cells with 10, 20, 50, or 100 μmol/l of NDGA for 7 days, the methylation-specific bands of E-cadherin gene still existed. The unmethylation-specific bands appeared after treatment with various concentrations of NDGA. In addition, NDGA dose-dependently reversed the hypermethylation status of E-cadherin gene in both cell lines.

To investigate whether demethylation correlated with gene reactivation, we examined the expression of E-cadherin gene at both RNA and protein levels. Untreated cell lines were negative for gene expression; however, after 7 days of treatment with NDGA, the cells expressed the transcript and protein. The restoration of E-cadherin expression in both cell types was accompanied by the appearance of unmethylated E-cadherin CpG island alleles in DNA from both cell types, as detected using an MSP technique. The pattern of changes of the mRNA and protein levels seemed to increase in a dose-dependent manner after treatment with NDGA (Fig. 2).

Effects of nordihydroguaiaretic acid on the in-vivo growth of human tumor xenografts

We also evaluated the antitumor activity of NDGA in the nu/nu mouse model, where mice received injections of MDA-MB-435 cells to induce tumors and were subsequently treated for 7 weeks with intraperitoneal injections of NDGA containing 2 mg of NDGA formulated in the Cremophor EL-based solvent three times a week. Control mice received solvent vehicle only. A significant reduction in tumor size was observed after treatment with NDGA, but not after treatment with vehicle solvent only (Fig. 3). The TGI (T/C) value for MDA-MB-435 tumor models was 37.3% ($\leq 42\%$), the minimum level for antitumor activity according to National Cancer Institute standards [20]. The health and well-being of the mice were assessed by recording their body weight at the beginning and end of the treatment. We observed little difference in body weight between control and NDGA-treated animals, indicating that NDGA produced minimal toxicity *in vivo* (Fig. 3).

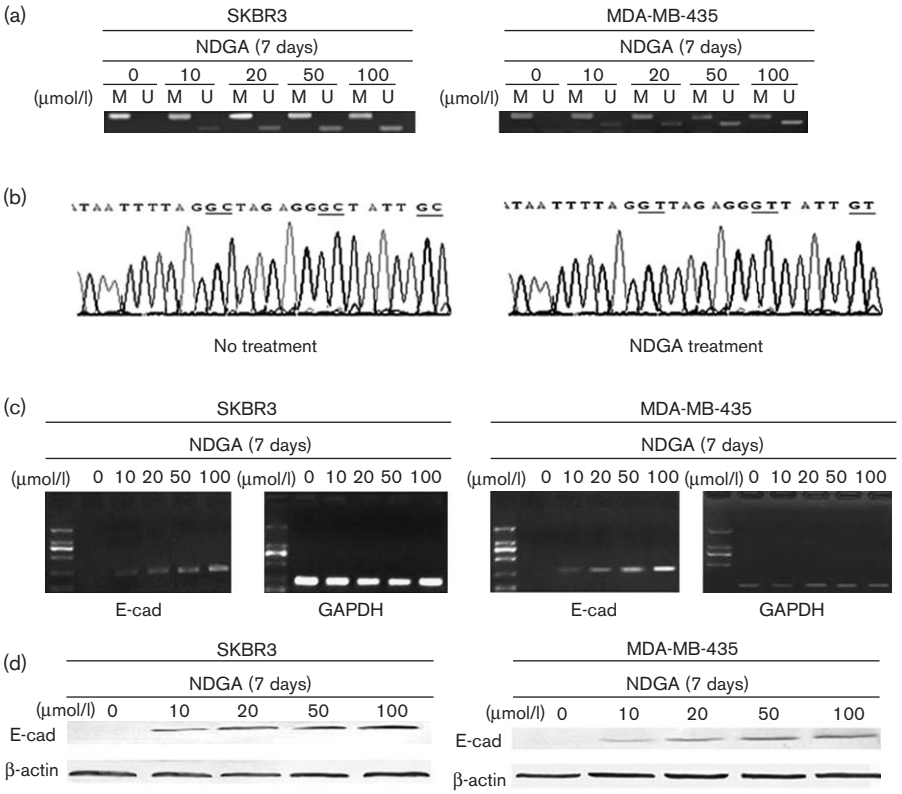
Effects of nordihydroguaiaretic acid on E-cadherin gene methylation status, mRNA, and protein expression *in vivo*

As shown in Fig. 4, the reversal of hypermethylation and reexpression of E-cadherin gene by NDGA in the human breast cancer xenograft models were similar to those in breast cancer cell lines. The relative amounts of protein E-cadherin were determined by immunohistochemistry in xenograft mice. When paraformaldehyde-fixed cells were treated with nonimmune serum, no cells were stained. When the cells were treated with an antibody to E-cadherin, almost all cells were positively immunostained in the NDGA-treated group compared with no immunostaining in the control group. These findings show that NDGA induced reexpression of E-cadherin in xenograft mice, which is likely owing to the demethylation of the E-cadherin gene (Fig. 4). From these data, we conclude that NDGA effectively reactivates the expression of E-cadherin gene *in vivo*.

Discussion

In breast cancer, there is substantial evidence demonstrating the importance of epigenetic mechanisms in the transcriptional regulation of critical tumor suppressor and growth regulatory genes [21]. As aberrant gene transcriptions resulting from epigenetic changes have been studied as a therapeutic target for agents that reverse this epigenotype, the idea of treating cancer patients with agents able to reestablish expression of tumor suppressor genes silenced by epigenetic mechanisms is currently being tested. Clinical trials using DNA methylation inhibitor and HDAC inhibitor or a combination of DNA methylation and HDAC inhibitors have already shown promising results in breast cancer [20], hematological neoplasms [22–24], cervix cancer [25], prostate cancer [26], lung cancer [27] etc. Nevertheless, the most

Fig. 2



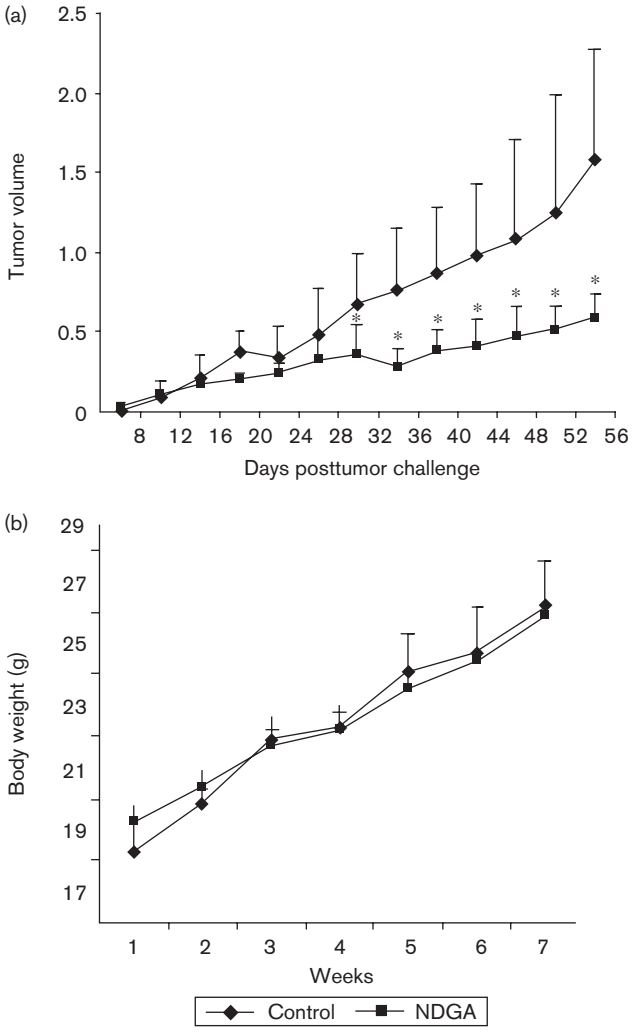
Alterations of methylation status, mRNA expression, and protein levels of E-cadherin gene in human breast cancer SKBR3 and MDA-MB-435 cells after treatment with NDGA for 7 days. (a) MSP was performed to assess methylation of the E-cadherin 5′ CpG island after treatment with different concentrations of NDGA at 7 days. Reactions specific for unmethylated DNA (U) or for methylated DNA (M) are indicated. (b) A sequenced region of MSP products showed that before NDGA treatment the cytosines of the two CpG dinucleotides remained unchanged after bisulfite treatment (left), consistent with methylation, whereas the cytosines of the two CpG dinucleotides were converted to uracil (amplified as thymidine) after NDGA treatment (right), consistent with a lack of methylation. (c) The mRNA levels of E-cadherin was determined with semiquantitative RT-PCR. The level of E-cadherin expression from each sample was further compared and normalized with glyceraldehyde 3-phosphate dehydrogenase. (d) Protein levels were determined with western blot analysis using β-actin as an internal control. Equal amounts (100 μg) of protein were loaded into each lane. Protein loading was normalized based on β-actin expression. MSP, methylation-specific PCR; NDGA, nordihydroguaiaretic acid.

broadly used DNA methyltransferase (DNMT) inhibitor, 5-aza-2′-deoxycytidine, clinically referred to as decitabine, has been shown to have toxic effects besides its demethylating properties and has been found to be mutagenic [28]. Therefore, there is an urgent need to find less toxic demethylating agents. The application of naturally occurring molecules in a clinical setting may be especially attractive, as these compounds have been shown to have limited toxicity.

Here, we report a natual compound NDGA, which may serve as an effective demethylating agent to reactivate the methylation-silenced critical tumor suppressor gene. Recently, several studies suggest that dietary lignans may be chemopreventive, potentially through antiestrogenic [29], antiangiogenic [30], proapoptotic [31], and antioxidant mechanisms [32]. The epidemiological data on the associations between serum and urine lignan concentrations and breast cancer risk are accumulating, and can be used to support the hypothesized role of lignans as breast cancer risk reducing agents [33]. However, the epidemiological data are not concurrent [34] and did not prove a causal relationship between lignan and breast cancer risk, thus leaving the role of lignans in primary and secondary breast cancer prevention largely open [35]. In this study, we chose NDGA as the model compound, as it is easily available and has already been well characterized in other model systems, to further elucidate the mechanism of lignan on cancer.

In this study, we use cultured SKBR3 and MAD-MB-435 human breast cancer cells, and a human breast MDA-MB-435 xenograft as experimental model systems to evaluate the effects of NDGA on DNA methylation and gene reactivation. It significantly inhibits the growth of breast cancer cells in both a time-dependent and

Fig. 3



Nu/nu mice carrying MDA-MB-435 xenograft tumors were treated with NDGA at doses of 100 mg/kg or with solvent alone for 7 weeks. (a) Effect of NDGA on growth of established human breast cancer, MDA-MB-435, in nude mice. MDA-MB-435 cells were injected into nu/nu mice on day 0. Treatment with NDGA began on day 8 with NDGA administered three times a week injected intraperitoneally (100 mg/kg). Values represent mean tumor volume ± SD for five animals per group. Tumor size was measured by calipers. Tumor volume significantly reduced for treatment groups (–■–) versus vehicle-treated controls (–◆–). * Indicates statistically significant ($P<0.05$). (b) Mice treated systemically with NDGA exhibit minimal toxicity. The mean change in body weight for NDGA-treated mice was not significantly different from that for the control mice. NDGA, nordihydroguaiaretic acid.

dose-dependent manner. In nude mice inoculated with human breast cancer MDA-MB-435 cells, NDGA was effective in reducing tumor incidence as well as reducing tumor burden compared with vehicle-treated controls. We demonstrated that NDGA reactivates the critical tumor suppressor genes E-cadherin 'silenced' by somatic CpG island hypermethylation and induces its expression in both breast cancer cells and in the athymic mouse model bearing MDA-MB-435 xenografts. We previously reported that NDGA induces demethylation and transcriptional reactivation at the mRNA and protein levels of the p16 genes in breast cancer cell line T47D (the data of this part have been submitted to the journal *Life Sciences*). This study confirms our previous findings, indicating that the demethylation effect of NDGA on tumor suppressor genes was not cell line-specific and gene-specific. In addition, in malignant glioma cell lines, Bian *et al.* demonstrated that NDGA inhibited 5-cytosine DNA methyltransferase activity [36]. Overall, taking all the above data into consideration, we may reasonably conclude that the anticancer properties of NDGA are related to its ability to demethylate and reactivate the expression of tumor suppressor genes.

To our knowledge, this report is the first to show that NDGA acts directly on activated silenced tumor suppressor genes *in vivo*. It is noteworthy that treatment with the systemic administration of NDGA showed excellent therapeutic efficacy without any signs of acute or chronic toxicity in mice in this study. The implications of this study are important because clinical toxicity is a major concern in the clinical applications of anticancer agents. Human tumor xenografts implanted s.c. in immunosuppressed mice have been widely used in preclinical anticancer drug development for the past 25 years. A retrospective study of compounds that have been tested in xenografts and in phase II trials revealed that 45% with activity against more than one-third of the xenografts tested also showed clinical activity [37]. These studies suggest that at least for existing cytotoxic anticancer drugs, the s.c. xenograft model is of predictive value. Human tumor xenografts are also particularly useful in determining pharmacodynamic markers of response for subsequent clinical application.

As a new and rapidly developing area of pharmacology, epigenetic therapy is a potentially very useful form of therapy, which uses drugs to correct epigenetic defects. As epigenetic changes are thought to underlie a wide range of diseases, the scope of epigenetic therapy is likely to expand. Taken together, the results of our study may not only help to further elucidate the precise mechanism of action of lignans in cancer prevention and therapy, but also indicate that NDGA may be a new and promising epigenetic modification compound safe and effective for the future of cancer chemoprevention and treatment. NDGA is promised to be a potentially useful therapy targeting one of the most common epigenetic abnormalities found in breast cancer and other malignancies.

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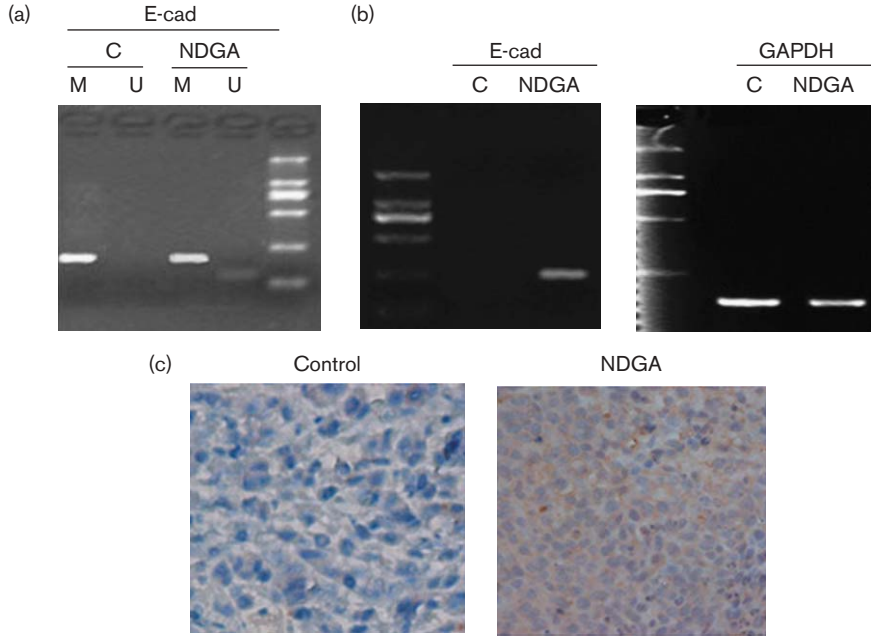
Fig. 4



Reactivation of E-cadherin expression in MDA-MB-435 cells *in vivo* by treatment with NDGA. (a) Representative gel analyses of methylation-specific PCR reactions on DNA from NDGA-treated group and control group. (b) Expression of E-cadherin mRNA determined using semiquantitative RT-PCR in NDGA group and control group with glyceraldehyde 3-phosphate dehydrogenase as an internal control. (c) Immunohistochemical staining for E-cadherin using specific human antibodies. Displayed are photomicrographs of MDA-MB-435 tumors subjected to immunohistochemical staining for E-cadherin after treatment with NDGA. C (control), complete loss of E-cadherin expression in MDA-MB-435 cells; NDGA, membrane expression of E-cadherin in cells (original magnification $\times 40$). M, methylated DNA; NDGA, nordihydroguaiaretic acid; U, unmethylated DNA.

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References

1 Saarinen NM, Warri A, Airio M, Smeds A, Makela S. Role of dietary lignans in the reduction of breast cancer risk. *Mol Nutr Food Res* 2007; **51**:857–866.

2 Cornwell T, Cohick W, Raskin I. Dietary phytoestrogens and health. *Phytochemistry* 2004; **65**:995–1016.

3 Arteaga S, Andrade-Cetto A, Cardenas R. Larrea tridentata (Creosote bush), an abundant plant of Mexican and US-American deserts and its metabolite nordihydroguaiaretic acid. *J Ethnopharmacol* 2005; **98**:231–239.

4 Fujimoto N, Kohta R, Kitamura S, Honda H. Estrogenic activity of an anti-oxidant, nordihydroguaiaretic acid (NDGA). *Life Sci* 2004; **74**:1417–1425.

5 Meyer GE, Chesler L, Liu D, Gable K, Maddux BA, Goldenberg DD, *et al.* Nordihydroguaiaretic acid inhibits insulin-like growth factor signaling, growth, and survival in human neuroblastoma cells. *J Cell Biochem* 2007; **102**:1529–1541.

6 Lee MK, Yang H, Ma CJ, Kim YC. Stimulatory activity of lignans from Machilus thunbergii on osteoblast differentiation. *Biol Pharm Bull* 2007; **30**:814–817.

7 Yang X, Yan L, Davidson NE. DNA methylation in breast cancer. *Endocr Relat Cancer* 2001; **8**:115–127.

8 Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, *et al.* E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 1991; **113**:173–185.

9 Meiners S, Brinkmann V, Naundorf H, Birchmeier W. Role of morphogenetic factors in metastasis of mammary carcinoma cells. *Oncogene* 1998; **16**:9–20.

10 Caldeira JR, Prando EC, Quevedo FC, Neto FA, Rainho CA, Rogatto SR. CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. *BMC Cancer* 2006; **6**:48.

11 Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res* 1998; **58**:95–101.

12 Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, *et al.* E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 1995; **55**:5195–5199.

13 Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 1994; **22**:2990–2997.

14 Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996; **93**:9821–9826.

15 Tamura G, Yin J, Wang S, Fleisher AS, Zou T, Abraham JM, *et al.* E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. *J Natl Cancer Inst* 2000; **92**:569–573.

16 Lee S, Kim WH, Jung HY, Yang MH, Kang GH. Aberrant CpG island methylation of multiple genes in intrahepatic cholangiocarcinoma. *Am J Pathol* 2002; **161**:1015–1022.

17 Sanceau J, Poupon MF, Delattre O, Sastre-Garau X, Wietzerbin J. Strong inhibition of Ewing tumor xenograft growth by combination of human interferon-alpha or interferon-beta with ifosfamide. *Oncogene* 2002; **21**:7700–7709.

18 Strickley RG. Solubilizing excipients in oral and injectable formulations. *Pharm Res* 2004; **21**:201–230.

19 Graff JR, Gabrielson E, Fujii H, Baylin SB, Herman JG. Methylation patterns of the E-cadherin 5′ CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem* 2000; **275**:2727–2732.

20 Arce C, Perez-Plasencia C, Gonzalez-Fierro A, de la Cruz-Hernandez E, Revilla-Vazquez A, Chavez-Blanco A, *et al.* A proof-of-principle study of epigenetic therapy added to neoadjuvant doxorubicin cyclophosphamide for locally advanced breast cancer. *PLoS ONE* 2006; **1**:e98.

21 Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. *Oncogene* 2002; **21**:5462–5482.

22 Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, *et al*. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. *Cancer Res* 2006; **66**: 6361–6369.

23 Garcia-Manero G, Kantarjian HM, Sanchez-Gonzalez B, Yang H, Rosner G, Verstovsek S, *et al*. Phase 1/2 study of the combination of 5-aza-2′-deoxycytidine with valproic acid in patients with leukemia. *Blood* 2006; **108**:3271–3279.

24 Yuan E, Haghighi F, White S, Costa R, McMinn J, Chun K, *et al*. A single nucleotide polymorphism chip-based method for combined genetic and epigenetic profiling: validation in decitabine therapy and tumor/normal comparisons. *Cancer Res* 2006; **66**:3443–3451.

25 Zambrano P, Segura-Pacheco B, Perez-Cardenas E, Cetina L, Revilla-Vazquez A, Taja-Chayeb L, *et al*. A phase I study of hydralazine to demethylate and reactivate the expression of tumor suppressor genes. *BMC Cancer* 2005; **5**:44.

26 Thibault A, Figg WD, Bergan RC, Lush RM, Myers CE, Tompkins A, *et al*. A phase II study of 5-aza-2′deoxycytidine (decitabine) in hormone independent metastatic (D2) prostate cancer. *Tumori* 1998; **84**:87–89.

27 Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J. Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs* 1997; **8**:358–368.

28 Juttermann R, Li E, Jaenisch R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci U S A* 1994; **91**:11797–11801.

29 Jackson-Grusby L, Laird PW, Magge SN, Moeller BJ, Jaenisch R. Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc Natl Acad Sci U S A* 1997; **94**: 4681–4685.

30 Horn-Ross PL, John EM, Canchola AJ, Stewart SL, Lee MM. Phytoestrogen intake and endometrial cancer risk. *J Natl Cancer Inst* 2003; **95**:1158–1164.

31 Wang L, Chen J, Thompson LU. The inhibitory effect of flaxseed on the growth and metastasis of estrogen receptor negative human breast cancer xenograftsis attributed to both its lignan and oil components. *Int J Cancer* 2005; **116**:793–798.

32 Hausott B, Greger H, Marian B. Naturally occurring lignans efficiently induce apoptosis in colorectal tumor cells. *J Cancer Res Clin Oncol* 2003; **129**:569–576.

33 Kinniry P, Amrani Y, Vachani A, Solomides CC, Arguiri E, Workman A, *et al*. Dietary flaxseed supplementation ameliorates inflammation and oxidative tissue damage in experimental models of acute lung injury in mice. *J Nutr* 2006; **136**:1545–1551.

34 Dai Q, Franke AA, Yu H, Shu XO, Jin F, Hebert JR, *et al*. Urinary phytoestrogen excretion and breast cancer risk: evaluating potential effect modifiers endogenous estrogens and anthropometrics. *Cancer Epidemiol Biomarkers Prev* 2003; **12**:497–502.

35 Boccardo F, Puntoni M, Guglielmini P, Rubagotti A. Enterolactone as a risk factor for breast cancer: a review of the published evidence. *Clin Chim Acta* 2006; **365**:58–67.

36 Webb AL, McCullough ML. Dietary lignans: potential role in cancer prevention. *Nutr Cancer* 2005; **51**:117–131.

37 Jiayou L, Xiuwu Bian. Changes of DNA methylation during differentiation of human glioma cells induced by NDGA. *Acta Academiae Medicinae Militaris Tertiae* 2003; **25**:283–286.