Prodigiosin induces the proapoptotic gene *NAG-1* via glycogen synthase kinase-3β activity in human breast cancer cells

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Abstract

Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene) is a bacterial metabolite that has anticancer and antimetastatic properties. However, the molecular mechanisms responsible for these abilities are not fully understood. Gene expression profiling of the human breast cancer cell line MCF-7 treated with prodigiosin was analyzed by cDNA array technology. The majority of the significantly modified genes were related to apoptosis, cell cycle, cellular adhesion, or transcription regulation. The dramatic increase of the *nonsteroidal anti-inflammatory drug-activated gene 1* (*NAG-1*) made this gene an interesting candidate regarding the possible mechanism by which prodigiosin induces cytotoxicity in MCF-7 cells. Our results show that prodigiosin triggers accumulation of the DNA-damage response tumor-suppressor protein p53 but that NAG-1 induction was independent of p53 accumulation. Moreover, prodigiosin caused AKT dephosphorylation and glycogen synthase kinase-3β (GSK-3β) activation, which correlated with NAG-1 expression. Prodigiosin-induced apoptosis was recovered by inhibiting GSK-3β, which might be due, at least in part, to the blockade of the GSK-3β–dependent up-regulation of death receptors 4 and 5 expression. These findings suggest that prodigiosin-mediated GSK-3β activation is a key event in regulating the molecular pathways that trigger the apoptosis induced by this anticancer agent. [Mol Cancer Ther 2007;6(1):362–9]

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Introduction

Cancer is a major public health problem in most developed countries. In particular, breast cancer is the most commonly diagnosed cancer among women and the second greatest cause of cancer deaths in women in most Western societies (1). Adjuvant chemotherapy has been proven to decrease the risk of relapse and cancer-related mortality in women with early-stage breast cancer. Moreover, chemotherapy is the treatment of choice for patients with hormone-insensitive and metastatic breast carcinomas (2). Unfortunately, most patients eventually develop recurrences due to the appearance of resistance to drugs after its reiterated administration. Thus, there is a need for new chemotherapeutic agents with novel and well-defined mechanisms of action.

The antimalarial, immunosuppressive, and procytotoxic bacterial metabolite prodigiosin (3) has recently been described as a novel anticancer and antimetastatic agent (4, 5). Prodigiosin promotes apoptosis in a wide variety of human cancer cell lines, including hematopoietic, gastrointestinal, and breast and lung cancer cells, with no marked toxicity in nonmalignant cells (4, 6–10). Prodigiosin triggers mitochondria-mediated apoptosis irrespective of multidrug resistance phenotype (9), and this apoptosis can be induced in p53-deficient cells (4). These features suggest an advantage to prodigiosin as an anticancer agent because they are very common phenomena that limit chemotherapy effectiveness. The molecular mechanism of prodigiosin cytotoxicity seems to be complex as it alters several biological processes of potential importance to cell viability. For example, prodigiosin has been shown to modulate intracellular pH through lysosomal alkalinization (11, 12), inhibit cell proliferation via G_1-S transition arrest (13), and interact with DNA inducing single- and double-strand breaks and topoisomerase I and II inhibition (14, 15).

Although the anticancer and proapoptotic activities of prodigiosin have been intensively studied, the molecular targets responsible for these properties have not yet been elucidated. To this end, we did gene expression profiling of MCF-7 breast cancer cells after treatment with prodigiosin. The most highly induced gene identified was *nonsteroidal anti-inflammatory drug-activated gene 1/growth differentiation factor 15* (*NAG-1*). NAG-1 is a secreted protein with homology to members of the transforming growth factor-β superfamily. Forced expression of NAG-1 in a variety of cell types has been related to cell cycle arrest and apoptosis (16–18). Many antitumorigenic compounds, including cyclooxygenase inhibitors (19), 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (20), retinoids (21), genistein (22), resveratrol (23), and vitamin D (24) have been shown to up-regulate NAG-1 expression. Several

mechanisms of NAG-1 induction have been described. NAG-1 expression can be induced in a p53-dependent (22–25) or p53-independent (26) manner. Other proteins that have been linked to NAG-1 expression are the early growth response gene-1 (27, 28), protein kinase C through nuclear factor-κB binding to NAG-1 promoter (29), and glycogen synthase kinase-3β (GSK-3β) through the phosphatidylinositol 3-kinase (PI3K)/AKT/GSK-3β pathway (30).

In the present study, we examine the molecular mechanism of prodigiosin-mediated induction of NAG-1 in MCF-7 cells and provide new insight into the molecular mechanism by which prodigiosin induces apoptosis in breast cancer cells.

Materials and Methods

Purification of Prodigiosin

Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene; Fig. 1) was extracted by shaking the *Serratia marcescens* 2170 cells with a mixture of methanol/1 N HCl (24:1). After centrifugation (68,006 × *g* for 15 min), the solvent of the supernatant was evaporated under vacuum. Atmospheric pressure liquid chromatography of the extract was done on silica gel with chloroform and methanol as solvents. The eluted pigmented fractions were pooled and the chloroform/methanol extract was vacuum evaporated, redissolved in H_2O, and lyophilized. The isolated pigment was redissolved in methanol and analyzed by electrospray ionization mass spectrometry using a VG-Quattro triple quadrupole mass spectrometer (Micromass, VG-Biotech, Manchester, United Kingdom). The isolated pigment was repurified by subsequent semipreparative high-performance liquid chromatography carried out on a Shimadzu instrument (Shimadzu, Kyoto, Japan). A Nucleosil C18 reversed-phase column (25,064 mm, 10 mm) was used with a 0 ± 100% linear gradient in 30 min [A, 0.01 mol/L ammonium acetate (pH 7); B, 100% acetonitrile]. The elution was monitored by both using diode-array UV detector (SPD-M10AVP Shimadzu) and electrospray ionization mass spectrometry. After repeated injections, the pooled fractions containing the major peak were vacuum evaporated, redissolved in H_2O, lyophilized, and characterized by electrospray ionization mass spectrometry and ^1H nuclear magnetic resonance. Electrospray ionization, *m/z* 324.4 (M+H)+, [$C_{20}H_{25}N_3O$ requires 323.4381 (molecular weight average)]. ^1H nuclear magnetic resonance (CD3OD, 500 MHz, ppm); 10.71 (m, NH), 8.54 (m, NH), 7.08 (s, 1H), 6.95 (s, 1H), 6.88 (m, 1H), 6.83 (m, 1H), 6.30 (m, 1H), 6.25 (s, 1H), 3.96 (s, 3H), 2.43 (t, 2H), 1.58 (s, 3H), 1.2 ± 1.4 (m, 6H), 0.91 (t, 3H). Stock solutions, with purity >95%, were prepared in methanol, and concentrations were determined by UV-Vis in 95% ethanol-HCl (ϵ_{535} = 11,2000/M cm).

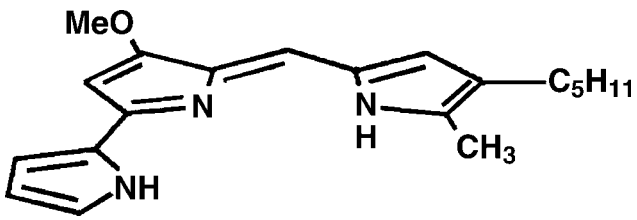

Figure 1. Side-on view of 2-methyl-3-pentyl-6-methoxyprodigiosene (prodigiosin) showing the planar arrangement of the three pyrrole rings.

Drugs

AR-A014418 was purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany).

Cell Lines and Culture Conditions

Human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM:Ham's F-12 (1:1; Biological Industries, Beit Haemek, Israel) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Carlsbad, CA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine. Cells were grown at 37°C in a 5% CO_2 atmosphere.

cDNA Array Analysis

MCF-7 cells were left untreated or treated with 0.5 μmol/L prodigiosin for 24 h. Atlas Pure Total RNA Labeling kit (Clontech, BD Biosciences, Palo Alto, CA) was used for total RNA isolation, polyadenylated RNA enrichment, and probe synthesis according to the manufacturer's instructions. Gene expression profiles were determined by hybridization to cDNA arrays (Atlas Human Cancer Array 1.2 from Clontech, BD Biosciences) and analyzed using AtlasImage 2.7 software. Three replicate arrays for each condition were averaged, and the composite arrays created were compared. Global normalization using the sum method, which adds the values of signal over background for all genes on the arrays to calculate the normalization coefficient, was used. The ratio corresponds to the expression of each gene relative to the untreated control cells (treated array gene adjusted intensity/control array gene adjusted intensity). A value of >2 or <0.5 is displayed in the ratio column when numerical values cannot be calculated because the gene signal on one array is at background level and is thus considered 0.

Quantitative Real-time Reverse Transcription-PCR

MCF-7 cells were treated with 0.5 μmol/L prodigiosin for 24 h. The inhibitor AR-A014418 (50 μmol/L) was added 30 min before prodigiosin treatment. Total RNA extraction was done using TRIzol reagent (Invitrogen Life Technologies), and cDNA synthesis was done using random hexamers and MuLV reverse transcriptase according to the manufacturer's instructions (Applied Biosystems, Warrington, United Kingdom). Each cDNA sample was analyzed for the expression of several genes using the fluorescent TaqMan 5′ nuclease assay. Oligonucleotide primers *nag-1* (*gdf-15*), *dr-4* (*tnfrsf10a*), *dr-5* (*tnfrsf10b*), *β-actin* (*actb*), and probes were purchased as Assay-on-Demand Gene Expression Products (Applied Biosystems). PCR assays were done using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Gene expression levels were normalized to β-actin, and relative mRNA expression was presented in relation to the control. Data were analyzed using Sequence Detector Software (version

1.9, Applied Biosystems) and are presented as mean ± SD of three independent experiments. For statistical analysis among treatment groups, ANOVA and least significant difference tests were done with the Statgraphics plus 5.1 statistical software.

Western Blot Analysis

MCF-7 cells were treated with several concentrations of prodigiosin for different times depending on the experiment. AR-A014418, when used, was added 30 min before prodigiosin treatment. Supernatants were collected, and cells were washed with PBS before addition of lysis buffer [85 mmol/L Tris-HCl (pH 6.8), 2% SDS, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.1 mmol/L phenylmethylsulfonyl fluoride]. Protein concentration was determined with BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as standard. Fifty micrograms of protein extracts were separated by 12% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were developed with primary antibodies according to the manufacturer's instructions. Antibodies were obtained from the following sources: anti–NAG-1/PTGF-β and anti–POLY(ADP)RIBOSE POLYMERASE were from Santa Cruz Biotechnology (Santa Cruz, CA); anti–CASPASE-8 was from PharMingen (BD Biosciences); anti-P53 was from Neomarkers (Fremont, CA); phosphorylated AKT (Ser^{473}) was from Cell Signaling Technology (Beverly, MA); and anti–β-ACTIN and anti-VINCULIN were from Sigma Chemical Co. (St. Louis, MO). Antibody binding was detected with the appropriate secondary antibodies conjugated to horseradish peroxidase, and signals were detected using the enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, United Kingdom). Vinculin was used as gel loading controls. Results shown are representative data obtained from three independent experiments.

Dominant Negative p53 Retrovirus Production and Infection of MCF-7 Cells

An amphotropic packaging cell line (Phoenix cells, a gift from Dr. Garry Nolan, Stanford University, Stanford, CA) was used to prepare dominant negative p53 retrovirus capable of infecting human MCF-7 cells. Eighteen hours before transfection, Phoenix cells were plated at 1×10^6 per 6-cm dish in 3 mL DMEM + 10% FBS. The cells were transfected with 10 μg pMSCV-IRES-GFP-p53dd or empty vector (gifts from Dr. James DeGregori, University of Colorado Health Sciences Center, Aurora, CO) using FuGENE 6 reagent (2 μL of FuGENE 6 per microgram of DNA). Twenty-four hours posttransfection, the medium on transfected Phoenix cells was changed to 3 mL fresh DMEM + 10% FBS. On this day, MCF-7 cells were plated at 2×10^5 per 6-cm dish in 3 mL DMEM + 10% FBS for viral infection. Forty-eight hours posttransfection, 3 mL of medium from transfected Phoenix cells were collected and filtered through a 0.45-μm filter to remove cellular debris. One milliliter of medium was removed and discarded from MCF-7 cells plated 24 h earlier. Three microliters of polybrene (5 mg/mL) were added to the remaining 2 mL medium on MCF-7 cells, and the plate was gently rocked to ensure mixing. Filtered viral medium (1 mL) was added dropwise to MCF-7 cells. Cells were allowed to incubate at 37°C for 24 h. Twenty-four hours postinfection, the medium on MCF-7 cells was refreshed with 3 mL DMEM + 10% FBS. Infected MCF-7 cells were fed and passaged as needed in DMEM + 10% FBS. pMSCV-IRES-GFP-p53dd contains the green fluorescent protein gene, which facilitated the identification of virally infected cells by flow cytometry. A pool of green fluorescent cells was recovered from infection with both empty viral vector and p53 dominant negative.

Cell Viability Assay

MCF-7 cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (31). Cells were plated in triplicate wells (2.5×10^4 per well) in 100 μL of growth medium in 96-well plates and incubated for 24 h. Then, cells were pretreated for 30 min with 50 μmol/L AR-A014418 before treatment with 1.4 μmol/L prodigiosin. After 24-h incubation, 10 μmol/L MTT (Sigma Chemical) was added to each well for an additional 4 h. The formazan precipitate was dissolved in 100 μL of isopropanol/1 N HCl (24:1), and absorbance at 570 nm was measured on a multiwell plate reader. Cell viability was expressed as a percentage of control and data are shown as the mean value ± SD of three independent experiments. Statistical analysis (ANOVA and least significant difference tests) was carried out with the Statgraphics plus 5.1. statistical software.

Results

Gene Expression Profiling Identifies *NAG-1* as a Prodigiosin Target Gene in MCF-7 Cells

To elucidate which genes are potentially involved in the cytotoxic cellular response of the anticancer agent prodigiosin (Fig. 1) in MCF-7 cells, changes in the expression profile of 1,176 genes on the Atlas Human Cancer Array 1.2 (BD Biosciences Clontech) were analyzed. MCF-7 cells were treated with 0.5 μmol/L prodigiosin for 24 h. This concentration of prodigiosin was used because it represents the IC_{25} (drug concentration that caused a cell viability decrease of 25% in cell viability) previously reported (9). Genes whose expression was up-regulated >2-fold or down-regulated >0.5-fold were considered to be significantly modulated by prodigiosin treatment (Table 1). Among the 20 up-regulated and 17 down-regulated genes, most of them were related to apoptosis, cell cycle, cellular adhesion, or transcriptional regulation. One particular gene, *NAG-1*, was the most highly induced after prodigiosin treatment. This gene, encoding a protein implicated in cell cycle blockage and apoptosis, was chosen for further study. Quantitative real-time reverse transcription-PCR and immunoblotting assays were done to confirm up-regulation of NAG-1 by prodigiosin treatment of MCF-7 cells. Total RNA and protein were prepared from MCF-7 cells treated with 0.5 μmol/L prodigiosin for 1, 8, and 24 h. NAG-1 mRNA levels

increased significantly in a time-dependent manner (79-fold increase at 24 h; Fig. 2A). A concomitant increase in NAG-1 protein was also observed starting at 4 h of prodigiosin treatment (Fig. 2B).

Prodigiosin-Mediated Induction of *NAG-1* Is p53 Independent

NAG-1 is a p53 target gene (17). However, the induction of NAG-1 by various compounds has been shown to be both p53 dependent and p53 independent. For example, resveratrol increases the cellular level of p53 in human colorectal cancer cells, thus promoting NAG-1 expression (23). We therefore examined p53 levels in MCF-7 and MDA-MB-231 breast cancer cells after prodigiosin treatment. MCF-7 cells were treated with 0.5 μmol/L prodigiosin for various times and p53 levels were determined by immunoblot analysis. Although a decrease in p53 levels at 4 and 8 h of treatment was observed, at 16 and 24 h of treatment, p53 protein levels increased, which correlated with increased NAG-1 protein levels (Fig. 3A). Moreover, when MCF-7 cells were treated with higher doses of prodigiosin corresponding to IC_{25}, IC_{50}, and IC_{75} values for 24 h, a parallel increase in p53 and NAG-1 protein levels was observed (Fig. 3B). However, p53 accumulation is not necessary for NAG-1 expression induced by prodigiosin because cells harboring mutant p53 (MDA-MB-231) treated with prodigiosin (doses corresponding to IC_{25}, IC_{50}, and IC_{75} values at 24 h for these cells) also express NAG-1 despite the lack of functional p53 (Fig. 2B). To further investigate the p53 independence of prodigiosin-mediated induction of NAG-1, we expressed a dominant negative

Table 1. Differentially expressed genes in MCF-7 cells after prodigiosin treatment

Gene name	Genbank no.	SwissProt no.	Ratio	Classification
Up-regulated genes				
Interferon induced transmembrane protein 1 (9-27) (IFITM1)	J04164	P13164	2.06	Cell cycle
Cyclin-dependent kinase inhibitor 1 (CDKN1A/p21)	U09579	P38936	>2	Cell cycle
Fms-related tyrosine kinase 1 (FLT1)	X51602	P17948	>2	Cell cycle
Junction plakoglobin (JUP)	M23410	P14923	>2	Cell adhesion
Ubiquitin-conjugating enzyme E2 17-kDa (UBE2A)	M74524	P49459	>2	Protein turnover
Purine-rich ssDNA-binding protein α (PURA)	M96684	Q00577	>2	Transcription regulator and DNA replication
Zinc finger protein 36 (ZFP36L1)	X79067	Q07352	>2	Transcription regulator
Integrin β_4 (ITGB4)	X53587	P16144	>2	Cell adhesion
HLA-G histocompatibility antigen, class I, G (HLA-G)	M32800	Q30182	>2	Cellular defense response
Procollagen (type III) N-endopeptidase (PCOLN3)	U58048	Q15779	>2	Cell cycle
Teratocarcinoma-derived growth factor 3 (TDGF3)	M96956	P13385	>2	Cell differentiation
Interleukin-1, β (IL1B)	K02770	P01584	>2	Cell cycle and apoptosis
Cell division cycle 34 (CDC34)	L22005	P49427	>2	Protein turnover and cell cycle
Nonsteroidal anti-inflammatory drug-activated gene 1/growth differentiation factor 15 (NAG-1/GDF15)	AF019770	Q99988	11.72	Cytokine activity, cell cycle and apoptosis
RAN binding protein 2 (RANBP2)	L41840	P49792	>2	Trafficking/targeting protein
Keratin 7 (KRT7)	X03212	P08729	>2	Cytoskeleton protein
IFN-stimulated exonuclease gene 20 kDa (ISG20)	U88964	O00586	>2	Cell cycle
Proteasome activator subunit 1 (PSME1)	L07633	Q06323	>2	Protein turnover
Eukaryotic translation elongation factor 1α1 (EEF1A1)	M27364	Q14222	2.22	Translation elongation
Insulin-induced gene 1 (INSIG1)	U96876	O15503	>2	Metabolism
Down-regulated genes				
Deleted in colorectal carcinoma (DCC)	X76132	P43146	<0.5	Cell cycle and apoptosis
v-myc myelocytomatosis viral oncogene homologue (MYC/c-myc)	V00568	P01106	0.36	Cell cycle
Notch homologue 4 (NOTCH4)	U95299	O00306	<0.5	Cell differentiation
v-abl Abelson murine leukemia viral oncogene homologue 2 (ABL2)	M35296	P42684	<0.5	Oncogenes
Prostaglandin E synthase (PTGES)	AF010316	O14684	<0.5	Metabolism
Prohibitin 2 (PHB2)	U72511	Q99623	<0.5	Transcription regulator
Guanylate kinase 1 (GUK1)	L76200	Q16774	<0.5	Metabolism
Rho GDP dissociation inhibitor (GDI) α (ARHGDIA)	X69550	P52565	0.44	Cell motility and apoptosis
TNF receptor-associated protein 1 (TRAP1)	U12595	Q12931	0.43	Apoptosis-associated proteins
Retinoic acid receptor, γ (RARG)	M24857	P13631	<0.5	Transcription regulator
Macrophage migration inhibitory factor (MIF)	M25639	P14174	0.47	Apoptosis-associated proteins
TIMP metallopeptidase inhibitor 1 (TIMP1)	X03124	P01033	<0.5	Cell cycle
Vitronectin (VTN)	X03168	P04004	<0.5	Cell adhesion
Keratin 8 (KRT8)	M34225	P05787	0.17	Cytoskeleton protein
Hemoglobin, α1 (HBA1)	V00491	P01922	<0.5	Trafficking/targeting proteins
Keratin 18 (KRT18)	M26326	P05783	<0.5	Cytoskeleton protein
Nonmetastatic cells 4, protein expressed in (NME4)	Y07604	O00746	<0.5	Metabolism

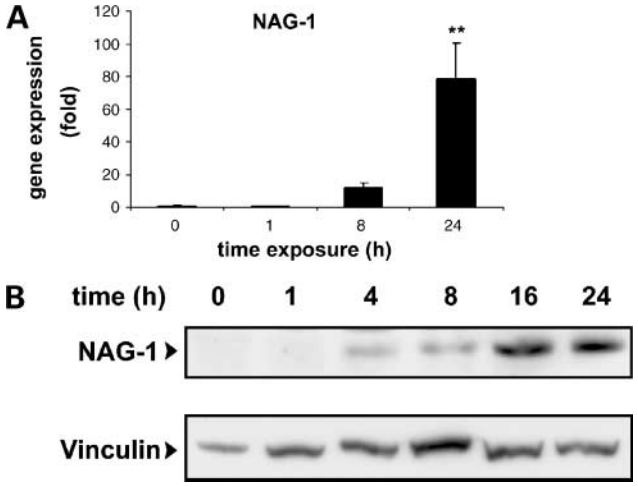

Figure 2. Effect of prodigiosin treatment on NAG-1 expression in MCF-7 cells. **A,** MCF-7 cells were treated for 1, 8, and 24 h with 0.5 μmol/L prodigiosin, and fold changes of gene expression with respect to control cells were determined by quantitative real-time reverse transcription-PCR. *Columns,* mean of triplicate experiments, normalized by using actin mRNA expression; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$, statistical significance among groups. **B,** time course analysis of protein levels in 0.5 μmol/L prodigiosin-treated MCF-7 cells subjected to immunoblotting with NAG-1 antibody. Vinculin is shown as a loading control, and representative blots of independent experiments are shown.

p53 (pMSCV-IRES-GFP-p53dd) in MCF-7 cells and analyzed NAG-1 protein levels. MCF-7 cells were infected with a retrovirus expressing a dominant negative form of p53. Western analysis for NAG-1 and p53 was done on a pool of virally infected cells. We compared protein levels in MCF-7 cells infected with the dominant negative–expressing retrovirus and MCF-7 cells infected with empty virus as a control. As shown in Fig. 3C, expression of dominant negative p53 in MCF-7 cells had no effect on NAG-1 expression. The blot was stripped and reprobed for an indicator of the efficiency of dominant negative p53 function: stabilization of p53. Infection of cells with pMSCV-IRES-GFP-p53dd results in stabilization of p53, indicating strong dominant negative p53 function in these cells. Taken together, although prodigiosin treatment of MCF-7 cells increases p53 protein levels, prodigiosin-mediated NAG-1 induction in breast cancer cells is p53 independent.

Prodigiosin-Mediated Induction of NAG-1 in MCF-7 Cells Is Dependent on GSK-3β Activity

The GSK-3β kinase has been implicated in *NAG-1* gene expression (30). To determine whether GSK-3β contributes to prodigiosin-mediated induction of NAG-1 in MCF-7 cells, experiments using a specific inhibitor of GSK-3β, AR-A014418, were done. MCF-7 cells were preincubated with 50 μmol/L AR-A014418 before treatment with 0.5 μmol/L prodigiosin. Prodigiosin-mediated induction of NAG-1 mRNA and protein was completely blocked after AR-A014418 treatment (Fig. 4). We also observed that prodigiosin caused dephosphorylation of AKT, a negative GSK-3β regulator. This could provoke GSK-3β activation, which may explain NAG-1 accumulation after prodigiosin treatment (Fig. 4B). To investigate whether GSK-3β activity was involved in the apoptotic phenotype induced by prodigiosin, cell viability experiments were done in the presence of AR-A014418. We observed that prodigiosin-mediated MCF-7 cell death was blocked by cotreatment with prodigiosin and AR-A014418 (Fig. 4C).

GSK-3β, through Regulation of Death Receptors 4 and 5 Expression, Is Implicated in Prodigiosin-Induced Apoptosis

It was previously shown that forced expression of NAG-1 significantly induced death receptor-4 (DR-4) and DR-5 induction in gastric cancer cells treated with the proapoptotic drug sulindac sulfide (32). Therefore, we analyzed gene expression of DR-4 and DR-5 in relation to GSK-3β activity in MCF-7 cells treated with prodigiosin (Fig. 5A). We observed a significant increase in DR-4 and DR-5

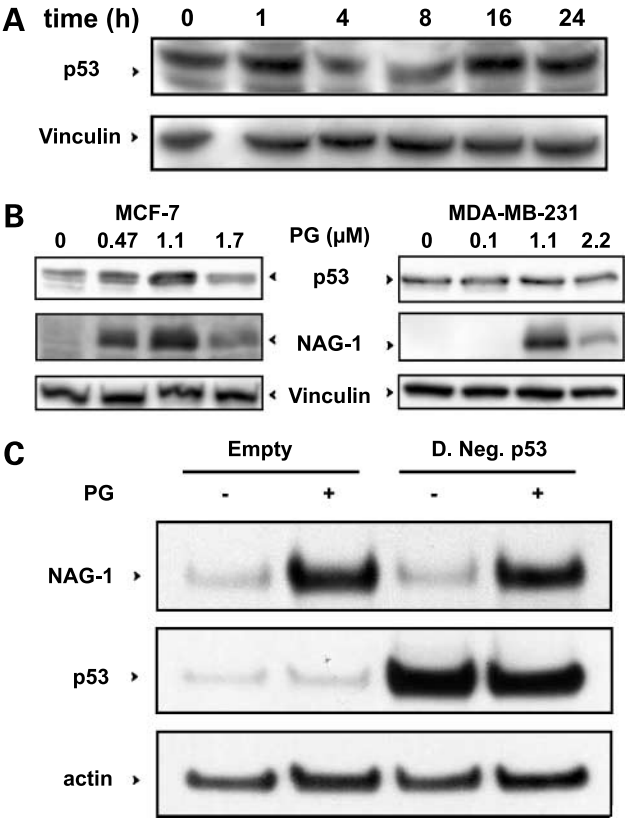

Figure 3. Analysis of p53 protein accumulation after prodigiosin exposure. **A,** cells were treated with 0.5 μmol/L prodigiosin for different times and cell lysates were subjected to Western blotting with p53 antibody. **B,** MCF-7 and MDA-MB-231 cells were incubated with different prodigiosin doses corresponding to their respective IC_{25}, IC_{50}, and IC_{75} values at 24 h and then subjected to immunoblotting for p53 and NAG-1 detection. Vinculin is shown as a loading control. **C,** MCF-7 cells were infected with a retrovirus expressing a dominant negative p53 (*D.Neg.p53*). A pool of infected cells was analyzed for NAG-1 protein levels after prodigiosin (*PG*) treatment (0.5 μmol/L). The same blot was stripped and developed with antibodies against NAG-1, p53, and β-actin as a loading control.

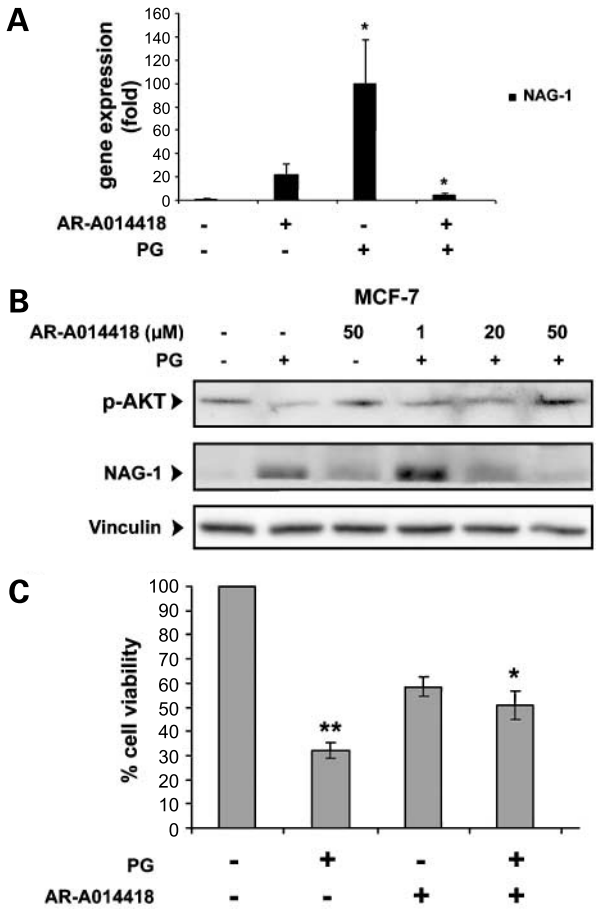

Figure 4. NAG-1 and cell viability regulation by GSK-3β. **A,** MCF-7 cells were exposed to 0.5 μmol/L prodigiosin for 24 h in the absence or presence of 50 μmol/L AR-A014418, and changes in gene expression (fold changes with respect to control cells) were evaluated by quantitative real-time reverse transcription-PCR. *Columns,* means of three independent experiments; *bars,* SD. *, $P < 0.05$, significant induction by prodigiosin or inhibition when combined with AR-A014418. **B,** after treating cells with 0.5 μmol/L prodigiosin for 24 h with or without 1, 20, or 50 μmol/L AR-A014418, cell lysates were collected for Western blot analysis using phosphorylated AKT (*p-AKT*), NAG-1, and vinculin antibodies. The latter is shown as a gel loading control, and representative blots of independent experiments are shown. **C,** cells were incubated with 1.4 μmol/L prodigiosin for 24 h alone or in the presence of 50 μmol/L AR-A014418, and cell viability was measured by the MTT assay. *Columns,* mean percentage of nontreated cells from triplicate experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$, statistical significance.

mRNA (5- and 13-fold, respectively) after 24 h of prodigiosin treatment. Importantly, this induction was inhibited in the presence of 50 μmol/L AR-A014418 (2- and 3-fold for DR-4 and DR-5, respectively), suggesting a critical role of GSK-3β in prodigiosin-mediated induction of DR-4 and DR-5. Finally, to evaluate whether death receptors are activated by prodigiosin treatment, the expression of their substrate, caspase-8, was analyzed (Fig. 5B). Caspase-8 was detected after 8 h of prodigiosin treatment, coinciding with cleaved poly(ADP)ribose polymerase, a caspase substrate indicative of apoptosis. These results show that apoptosis via the death receptor extrinsic pathway is active in these cells. Together, these results suggest that prodigiosin-induced apoptosis is mediated by GSK-3β and that caspase-8 activation through DR-4 and DR-5 might explain, at least in part, this phenomenon.

Discussion

The aim of this study was to identify genes that undergo a change in expression in response to prodigiosin treatment to determine its mechanism of action. This information is used to aid the progress of this treatment to the clinic. The majority of the significantly modified genes in response to prodigiosin treatment, revealed by cDNA array technology, were related to apoptosis, cell cycle, cell adhesion, or transcriptional regulation. We focused our study on the most highly modified gene, *NAG-1*. Our results support that prodigiosin treatment induces accumulation of the DNA-damage response protein p53 but that prodigiosin-mediated NAG-1 expression is p53 independent. Inactivation of the prosurvival pathway PI3K/AKT in MCF-7 cells was observed after prodigiosin treatment. Finally, NAG-1 and DR-4 and DR-5 expressions were abrogated by GSK-3β inactivation, as well as prodigiosin-induced apoptosis, suggesting that this kinase might be a key regulator of the prodigiosin cytotoxic effect.

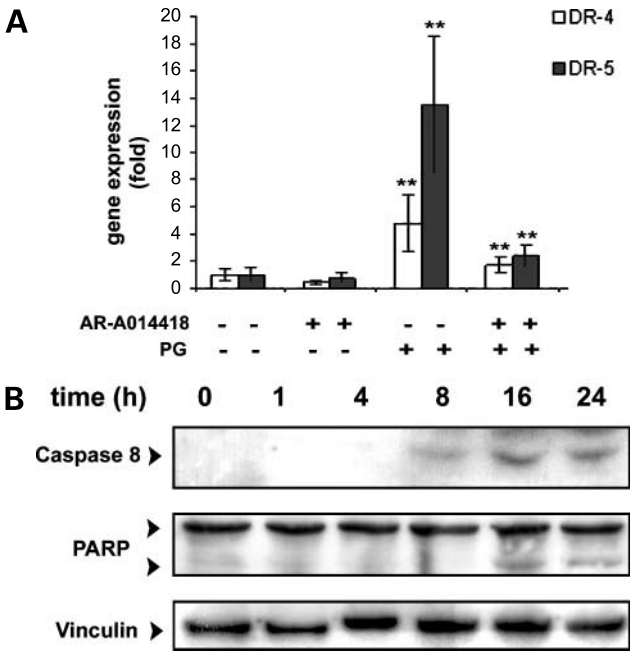

Figure 5. Analysis of apoptosis-related proteins after prodigiosin treatment. **A,** mRNA of MCF-7 cells, nontreated or treated with 50 μmol/L AR-A014418 before 24-h treatment with 0.5 μmol/L prodigiosin, was extracted. DR-4 and DR-5 levels were quantified by quantitative real-time reverse transcription-PCR. *Columns,* mean of triplicate experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$, statistical significance. **B,** cells were exposed to 0.5 μmol/L prodigiosin and a time course analysis of caspase-8, poly(ADP)ribose polymerase (*PARP*), and vinculin proteins was done by immunoblotting. Vinculin is shown as a gel loading control, and representative blots of independent experiments are shown.

Many chemotherapeutic agents currently used in the clinic induce accumulation of the tumor-suppressor protein p53, a key protein signaling growth arrest and apoptosis in response to DNA damage (33). In this regard, prodigiosin was previously described to intercalate to the DNA provoking topoisomerase I and II inhibition and, consequently, DNA cleavage (15). Moreover, accumulation of functional p53 protein and gene expression of the transforming growth factor-β family member NAG-1 after prodigiosin treatment were observed. NAG-1 can be induced in response to both p53-dependent and p53-independent apoptotic signaling events caused by DNA damage (18). Thus, one of the mechanisms of action of prodigiosin seems to be to cause DNA damage, which, in turn, triggers p53 accumulation. However, the ability of prodigiosin to induce apoptosis in cells with deficient p53 (4) indicates that p53 signaling after prodigiosin treatment is not essential for prodigiosin-induced cell death. This may confer prodigiosin an advantage in front of other chemotherapeutic agents that need functional p53 to provoke its cytotoxic effect because this protein is frequently mutated in most human cancers, which is related to poor prognosis (34).

Similar to prodigiosin, many cytotoxic agents have been reported to induce NAG-1 overexpression (19–23), but the exact mechanism by which NAG-1 triggers apoptosis is still poorly understood. NAG-1 induces morphologic changes followed by reduced cell adhesion and cell detachment in prostate cancer cells before undergoing apoptosis (16). Cell anchorage is not merely a structural feature of the cell but mediates pivotal survival signals into the cytoplasm; therefore, disturbance of cell anchorage frequently leads to the initiation of cell death by apoptosis, a process called anoikis (35). Cells treated with prodigiosin undergo progressive morphologic changes, cell detachment, and reorganization of actin microfilaments (7). In addition, the antimetastatic effect of prodigiosin is due to the inhibition of tumor invasion, which include the inhibition of cell adhesion and motility in a RhoA-dependent manner and suppression of matrix metalloproteinase-2 ability (5). Likewise, many of the genes regulated by prodigiosin, shown by the cDNA array experiments, are related to cellular adhesion, motility, or cytoskeleton structure. Taken together, these data suggest that one of the roles for NAG-1 in prodigiosin-induced apoptosis might be through morphologic changes leading to cell detachment, which then leads to prodigiosin-induced cell death.

The PI3K/AKT/GSK-3β signaling pathway has been shown to regulate NAG-1 expression in human colorectal carcinoma cells (30). The serine/threonine kinase AKT plays a key role in protecting cells from apoptosis through the phosphorylation of diverse downstream targets (36). Indeed, the blockade of this pathway sensitizes cells to various apoptotic stimuli, such as the response to the DNA-damaging agent doxorubicin in cancer cells in which the PI3K/AKT pathway is constitutively activated (37). GSK-3β is a negatively regulated AKT target whose overexpression has been shown to induce apoptosis whereas dominant-negative GSK-3β prevented apoptosis after inhibition of PI3K in prostate cancer cells (38). The AKT dephosphorylation caused by prodigiosin treatment allows GSK-3β activation leading to NAG-1 expression. This seems to be one of the molecular signaling events responsible for the apoptosis induced by prodigiosin. This was corroborated because GSK-3β pharmacologic inactivation recovered cell viability, suggesting a crucial role for GSK-3β in prodigiosin-induced apoptosis. Moreover, prodigiosin treatment induced expression of DR-4 and DR-5, which was reduced when GSK-3β was inhibited. These membrane receptors activate the initiator caspase-8, leading to apoptosis signaling through the extrinsic pathway (39). However, DR induction of GSK-3β via NAG-1 remains to be elucidated. NAG-1 cDNA transfection into gastric cancer cells significantly induced apoptosis and DR-4 and DR-5 expressions (33), suggesting a novel NAG-1 signaling pathway that may regulate DR expression.

In summary, the molecular mechanisms of the antitumorigenic potential of prodigiosin is effected by multiple events, giving rise to apoptosis. GSK-3β activation through inhibition of the PI3K/AKT pathway seems to be the most crucial event leading to prodigiosin apoptotic effect. GSK-3β–dependent expression of DR-4 and DR-5 may be through NAG-1 could explain, at least in part, prodigiosin-induced cell death. Furthermore, secretion of NAG-1 provides a route through which molecular signals caused by a cytotoxic agent can be communicated to the neighboring cells, and, thus, amplifying the effect of the cytotoxic agent. Altogether, these results point to prodigiosin as an attractive candidate for chemotherapy, especially in tumors with mutated p53 and activated PI3K/AKT/GSK3β pathway.

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