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The anticancer agent prodigiosin induces p21$^{WAF1/CIP1}$ expression via transforming growth factor-beta receptor pathway

Vanessa Soto-Cerrato [a], Francesc Viñals [b,c], James R. Lambert [d], Ricardo Pérez-Tomás [a,*]

[a] Department of Pathology and Experimental Therapeutics, Cancer Cell Biology Research Group, Universitat de Barcelona, Barcelona, Spain
[b] Laboratori de Recerca Translacional, ICO-IDIBELL, L'Hospitalet de Llobregat, Barcelona, Spain
[c] Departament de Ciències Fisiològiques II, Campus de Bellvitge, Universitat de Barcelona, Barcelona, Spain
[d] Department of Pathology, University of Colorado Denver and Health Science Center, Aurora, CO, USA

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ABSTRACT

The anticancer agent prodigiosin has been shown to act as an efficient immunosuppressant, eliciting cell cycle arrest at non-cytotoxic concentrations, and potent proapoptotic and antimetastatic effects at higher concentrations. Gene expression profiling of MCF-7 cells after treatment with a non-cytotoxic concentration of prodigiosin showed that expression of the p21$^{WAF1/CIP1}$ gene, a negative cell cycle regulator was induced. In this study, we show that prodigiosin induces p21 expression leading to cell cycle blockade. Subsequently, we attempted to elucidate the molecular mechanisms involved in prodigiosin-mediated p21 gene expression. We demonstrate that prodigiosin induces p21 in a p53-independent manner as prodigiosin induced p21 in cells with both mutated and dominant negative p53. Conversely, the transforming growth factor-beta (TGF-β) pathway has been found to be necessary for p21 induction. Prodigiosin-mediated p21 expression was blocked by SB431542, a TGF-β receptor inhibitor. Nevertheless, this pathway alone is not enough to induce p21 expression. The TGF-β family member (nonsteroidal anti-inflammatory drug)-activated gene 1/growth differentiation factor 15 (NAG-1) may activate this pathway, as it has previously been suggested to signal through the TGF-β pathway and is overexpressed in response to prodigiosin treatment. We show that NAG-1 colocalizes with TGF-β receptor type I, suggesting a possible interaction between them. Taken together, these results suggest the TGF-β pathway is required for induction of p21 expression after prodigiosin treatment of MCF-7 cells.

1. Introduction

The bioactive secondary metabolite prodigiosin (2-methyl-3-pentyl-6-methoxyprodiginine) belongs to a family of tripyrrole red pigments produced by both Gram-negative and Gram-positive bacteria [1]. Prodigiosin is effective as an immunosuppressant at non-cytotoxic concentrations [2]. Higher levels lead to anticancer and antimetastatic effects [3,4]. Prodigiosin

* *Corresponding author at*: Department of Pathology and Experimental Therapeutics, Cancer Cell Biology Research Group, Universitat de Barcelona. Pavelló Central, 5a planta, LR 5101C/Feixa Llarga s/n, E 08907 L'Hospitalet de Llobregat, Barcelona, Spain. Tel.: +34 934024288; fax: +34 934029082.
E-mail address: rperez@ub.edu (R. Pérez-Tomás).

Abbreviations: GSK-3β, glycogen synthase kinase-3 beta; FBS, fetal bovine serum; IC_{25}, inhibitory concentration 25; MAPK, mitogen-activated protein kinase; MTT, methyl-thiazole-tetrazolium; NAG-1/GDF-15, (nonsteroidal anti-inflammatory drug)-activated gene 1/growth differentiation factor 15; p21, p21$^{WAF1/CIP1}$; PG, prodigiosin; TGF-β, transforming growth factor-beta; TGF-βRI, TGF-β receptor type I.

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provokes cell death in a broad range of human cancer cell lines [5–8]. It induces mitochondria-mediated apoptosis irrespective of multidrug resistance phenotype [9,10]. Resistance is a common phenomenon that reduces the effectiveness of chemotherapy. Interestingly, prodigiosin has multiple mechanisms of action. However, the contribution of each mechanism to the observed effects is still unclear. Prodigiosin reversibly disrupts the pH gradient between lysosomes and cytoplasm [11], induces G_1-S transition arrest [12], causes DNA fragmentation and topoisomerases inhibition [13,14], induces GSK-3β activation [15] and exerts an uncoupling effect on the electron chain transport of protons to mitochondrial ATP synthase [16].

Transforming growth factor-beta (TGF-β) family cytokines regulate many physiological processes such as cell proliferation, differentiation, adhesion, matrix production, motility and apoptosis [17,18]. TGF-β members exert their biological effects by signaling through membrane-bound receptors. The best characterized is the TGF-β/Smad pathway. Binding of TGF-β family members to type II receptors (TβRII) leads to the formation of a heterodimeric cell surface receptor complex together with a type I receptor (TβRI). The latter is phosphorylated by TβRII and thus activated. It subsequently phosphorylates a receptor-regulated Smad, allowing this protein to associate with Smad-4 and translocate into the nucleus. Once in the nucleus, the Smad complex activates transcription of target genes [19]. Other signaling pathways have also been implicated downstream from the TGF-β receptors, including several Smad-independent mitogen-activated protein kinase (MAPK) pathways [20].

The growth-inhibitory effect of TGF-β signaling in epithelial cells is the consequence of the activation of a cytostatic gene response program that includes the down-regulation of the c-Myc and Id family of transcription factors, and the activation of $p15^{INK4b}$ and $p21^{WAF1/CIP1}$ (p21) cyclin-dependent kinase inhibitors [21]. p21 mainly inhibits the activity of cyclin/cdk2 complexes [22] and negatively regulates cell cycle progression after cell exposure to different stimuli such as DNA-damaging agents [23]. Apart from the tumor suppressor p53, a variety of other factors (including Sp1/Sp3, Smads, Ap2, STAT, BRCA1, E2F-1/E2F-3, and CAAT/enhancer binding protein α and β) are known to activate p21 transcription [24].

The TGF-β family member (nonsteroidal anti-inflammatory drug)-activated gene 1/growth differentiation factor 15 (NAG-1/GDF15) is a secreted protein thought to activate the TGF-β signaling pathway inducing cell cycle arrest [25] or apoptosis in many different cell types [26,27]. Many anti-tumorigenic compounds, such as cyclooxygenase inhibitors [28], retinoids [29], genistein [30], resveratrol [31], and vitamin D [32], among others, have been found to up-regulate its expression. Prodigiosin has recently been reported to induce NAG-1 expression, death receptors 4 and 5 and apoptosis in breast cancer cells through glycogen synthase kinase-3 beta (GSK-3β) activation [15].

In this report, we demonstrate p21 induction and subsequent cell cycle arrest in MCF-7 breast cancer cells following prodigiosin treatment. Identification of TGF-β signaling as an essential molecular pathway responsible for prodigiosin-mediated p21 expression is reported and new insights into the role of this pathway on prodigiosin-induced cytostatic effects are provided.

2. Materials and methods

2.1. Drugs and reagents

2-Methyl-3-pentyl-6-methoxyprodigiosene, also called prodigiosin, was purified from *Serratia marcescens* 2170, as previously described [5]. Stock solutions were prepared in methanol and their concentrations were then determined by UV–vis in 95% EtOH–HCl (ε_{535} = 112,000/M cm). SB431542 (Cat# 1614) and AR-A014418 (Cat# 361546) were purchased from Tocris (Ellisville, MO) and Calbiochem (EMD Biosciences, Darmstadt, Germany), respectively.

2.2. 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 F12 (1:1) (Biological Industries, Beit Haemek, Israel) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO BRL, Invitrogen life technologies, Carlsbad, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, all from Biological Industries. C2C12 mouse cells were cultured in DMEM containing 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin sulphate. Cells were grown at 37 °C in a 5% CO_2 atmosphere.

2.3. cDNA array analysis

Gene expression was analyzed by hybridization to cDNA arrays (Atlas™ Human Cancer Array 1.2 from Clontech, BD Biosciences, Palo Alto, CA) as previously described [15]. Briefly, cells (1.5×10^7 in 30 ml) were untreated (control) or treated with 0.5 μM prodigiosin for 24 h. An Atlas™ Pure Total RNA Labeling kit (Clontech, BD Biosciences) was used for total RNA isolation, poly A^+ RNA enrichment and probe synthesis. Hybridization to cDNA arrays was performed, films were scanned and image analysis was carried out with BD AtlasImage™ 2.7 (Clontech, BD Biosciences).

2.4. Quantitative real-time RT-PCR

Cells (5×10^5 cells/ml) were exposed to 0.5 μM prodigiosin for 24 h. When the inhibitors AR-A014418 (50 μM) and SB431542 (20 μM) were used, they were added 30 min before prodigiosin treatment. Total RNA extraction was performed using TRIzol® Reagent (Invitrogen life technologies). The RNA pellet was washed in 75% ethanol, dissolved in H_2O, and cDNA synthesis (1 μg RNA/50 μl) was performed using random hexamers and MuLV RT, according to the manufacturer's instructions (Applied Biosystems, Warrington, UK). Each cDNA sample was analyzed for the expression of several genes using the fluorescent TaqMan 5′ nuclease assay. Oligonucleotide primers p21 (CDKN1A, Cat# Hs00355782_m1), death receptor (DR) 4 (TNFRSF10A, Cat# Hs00269492_m1), DR-5 (TNFRSF10B, Cat# Hs00187196_m1), beta actin (ACTB, Cat# Hs99999903_m1) and probes were purchased as Assay-on-Demand Gene Expression Products (Applied Biosystems). The 5′ nuclease assay PCRs were performed using the ABI PRISM 7700 Sequence Detection System for thermal cycling and real-time fluorescence measurements (Applied Biosystems). Each 50 μl reaction

consisted of 1X TaqMan Universal PCR MasterMix (PE Biosystems); 1X Assay-on-Demand mix containing forward primer, reverse primer, and a TaqMan quantification probe (Applied Biosystems); and a 100 ng cDNA template. Reaction conditions consisted of an initial step at 92 °C for 10 min, then 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The gene expression levels obtained were normalized by mRNA expression of actin. The relative mRNA expression was then presented in relation to the control. Data were analyzed using ''Sequence Detector Software'' (SDS Version 1.9, Applied Biosystems) and were presented as the mean ± S.D. of three independent experiments. For the statistical analysis among treatment groups, ANOVA and LSD tests were performed with the Statgraphics plus 5.1 statistical package. $P < 0.05$ and $P < 0.01$ were represented with * and **, respectively.

2.5. Western blotting

Cells (5×10^5 cells/ml) were exposed to several prodigiosin concentrations for different times, depending on the experiment. When used, the inhibitors AR-A014418 and SB431542 were added 30 min before prodigiosin treatment. Supernatants, with detached cells were then collected, centrifuged, pooled with the cells on the plate and washed in PBS prior to the addition of a lysis buffer (85 mM Tris–HCl pH 6.8, 2% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride). The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) as a standard. Fifty micrograms of protein extracts were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked in 5% dry milk diluted in TBS–Tween (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h and then incubated overnight with primary antibodies, according to the manufacturer's instructions. Antibodies were obtained from the following sources: anti-actin (Cat# sc-1616) was from Santa Cruz Biotechnology, (Santa Cruz, CA); anti-p21 (Cat#OP-64) was from Calbiochem (La Jolla, CA); anti-p53 (Cat# MS-186-P1) was from Neomarkers (Fremont, CA); phospho-smad-2 Ser465/467 (Cat# 3101) was from Cell Signaling Technology (Beverly, MA); anti-vinculin (Cat# V-4505) was from Sigma (St Louis, MO). Antibody binding was detected with goat anti-rabbit, goat anti-mouse (Bio-Rad Laboratories, Hercules, CA) or donkey anti-goat (Santa Cruz Biotechnology) immunoglobulin G (IgG) secondary antibodies conjugated to horseradish peroxidase and the ECL detection kit (Amersham, Buckinghamshire, UK). Actin or vinculin were used as gel loading controls. The results shown are representative of Western blot data obtained from at least three independent experiments with identical observations.

2.6. [^{3}H]-Thymidine incorporation assay

MCF-7 cells (5×10^5 cells/ml) were seeded in a 24-well plate. Cells were incubated in complete medium in the absence or presence of the indicated concentrations of prodigiosin and with 1 μCi of [^{3}H] thymidine ([6-^{3}H] thymidine) (0.5 Ci/mmol, Amersham Pharmacia Biotech) for 24 h. Cells were washed twice in cold 5% TCA and lysed with 0.1 M NaOH. The lysates were mixed with 5 ml scintillation buffer. Radioactivite counts were then measured using a scintillation counter (Beckman). The mean of triplicate experiments and standard deviations are shown.

2.7. Flow cytometry

MCF-7 cells (1×10^6) were plated in 10 cm dishes 16 h prior to treatment with prodigiosin or methanol control. After a 24 h-treatment, cells were trypsinized, collected and centrifuged at 1500 rpm for 5 min. Then, cells were resuspended in 1.5 ml saponin/PI solution (0.3% saponin (w/v), 2.5% PI (w/v), 0.1 mM EDTA, 10 μg/ml RNase in PBS) and incubated overnight in the dark. FACS analysis was performed using a Beckman Coulter FC500 flow cytometer. ModFit LT software (Verity Software House, Topsham, ME) was used for doublet exclusion and cell cycle analysis.

2.8. Dominant negative p53 MCF-7 cells

Dominant negative p53 retrovirus production and infection of MCF-7 were performed as previously described [15].

2.9. Immunocytochemistry

Cells cultured in a 24-well plate containing glass coverslips (1.25×10^5 cells in 250 μl) were incubated with 0.5 μM prodigiosin for 24 h. When using the inhibitor SB431542, a concentration of 20 μM was added 30 min before prodigiosin treatment. Cells were then washed twice with PBS and fixed with 4% paraformaldehyde for 20 min. Fixed cells were permeabilized with 0.2% Triton X-100 and then blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Cells were incubated overnight at 4 °C with anti-NAG-1/PTGF-β (1:50 dilution, Cat# sc-10603) and anti-TGF-β Receptor I (1:50 dilution, Cat# sc-398) antibodies, both from Santa Cruz Biotechnology or 1 h at room temperature with Smad 2/3 (1:50 dilution, Cat#610843) from Pharmingen, BD Biosciences, Palo Alto, CA. The cells were washed with PBS containing 3% BSA and incubated with Alexa Fluor® 488-conjugated donkey anti-goat (Cat# A11055, Molecular Probes, Invitrogen) and/or Fluorolink™ Cy™ 3-labelled goat anti-rabbit (Cat# PA43004, Amersham Biosciences, Buckinghamshire, UK) IgGs at 1:400 dilution for 1 hour. Finally, a 15-min incubation with TO-PRO®-3 iodide (1:6000 dilution, Cat# T3605, Molecular Probes, Invitrogen) was performed and coverslips were placed on the slides using Immunofluore mounting medium (MD Biomedicals, Aurora, OH). The immunofluorescent images were captured using a Leica TCS SL spectral confocal microscope. Representative images from three independent experiments are shown.

2.10. Cell viability assay

Cell viability was determined using the methyl-thiazole-tetrazolium (MTT) assay [33]. Cells were plated in triplicate wells (2.5×10^4 cells/well) in 100 μl of growth medium in 96-well plates and allowed to grow for 24 h. Cells were pre-treated for 30 min with 20 μM SB431542 prior to 1.4 μM prodigiosin treatment. After 24 h incubation, 10 μM of MTT (Sigma

Chemical Co., St. Louis, MO) was added to each well for an additional 4 h. The blue MTT formazan precipitate was dissolved in 100 μl of isopropanol: 1N HCl (24:1). The absorbance at 570 nm was measured on a multiwell plate reader. Cell viability was expressed as a percentage of the control and data are shown as the mean value ±S.D. of three independent experiments. Statistical analysis (ANOVA and LSD tests) was carried out with the Statgraphics plus 5.1. statistical package. $P < 0.05$ and $P < 0.01$ were represented with * and **, respectively.

3. Results

3.1. Cell cycle arrest and p21 induction after prodigiosin treatment

cDNA array experiments analyzing differential gene expression after prodigiosin treatment were performed in our laboratory in order to identify the molecular targets of this anticancer drug [15]. MCF-7 cells were treated with 0.5 μM prodigiosin (IC_{25} at 24 h, the drug concentration that caused a cell viability decrease of 25% in the cell population [9]). The cell-cycle regulator protein p21 was identified among the most modulated genes. It was then selected for validation by more accurate methods, including quantitative real time RT-PCR and Western blot assays (Fig. 1). MCF-7 cells were treated with 0.5 μM prodigiosin for different time periods. p21 mRNA levels significantly increased, especially after 24 h of treatment (levels were 35-fold higher than in non-treated cells) (Fig. 1A). We also observed a time-dependent increase in p21 protein levels, which was significant from 16 h of drug treatment (Fig. 1B).

Because p21 is a cyclin dependent kinase inhibitors, we investigated the effect of prodigiosin on cell proliferation and cell cycle in MCF-7 cells. To determine the effect of prodigiosin on MCF-7 cell proliferation, ^{3}H-thymidine incorporation experiments were assessed. Different doses of prodigiosin

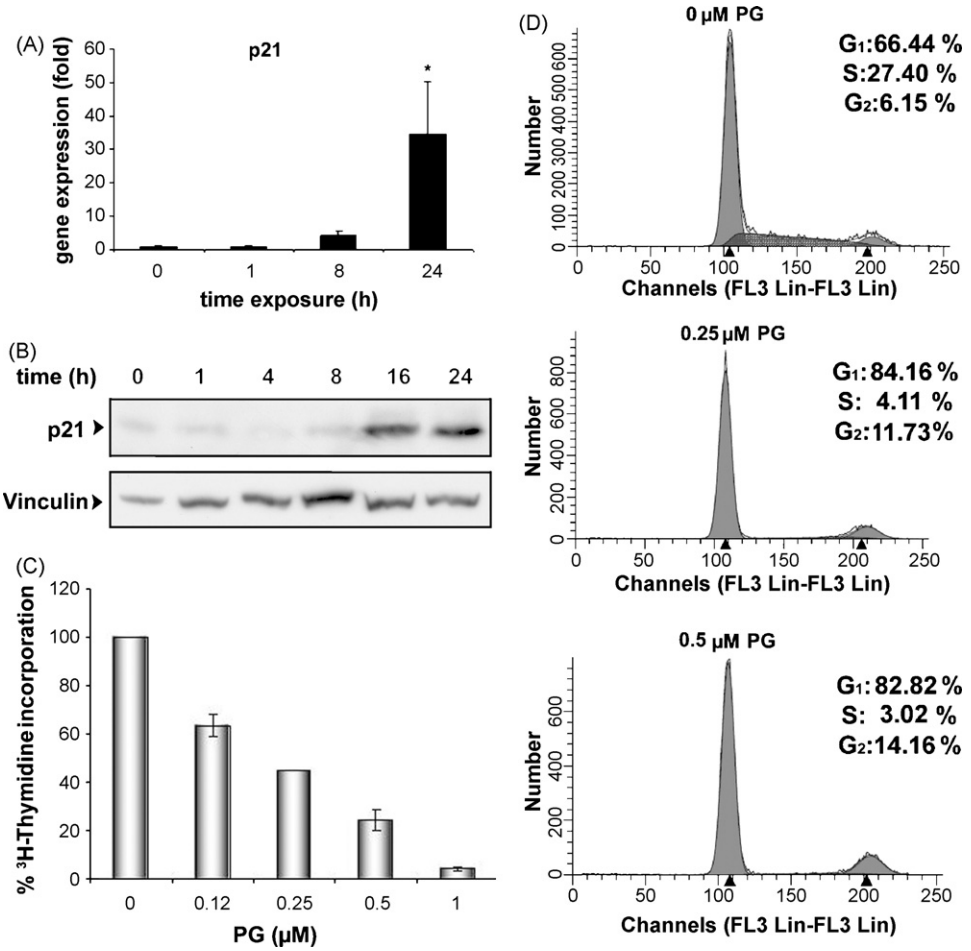

Fig. 1 – The effect of prodigiosin treatment on p21 expression and the cell cycle regulation of MCF-7 cells. (A) MCF-7 cells were treated for 1, 8 and 24 h with 0.5 μM of prodigiosin, and fold changes in gene expression with respect to control cells were determined by quantitative real time reverse transcription-PCR. The values are expressed as the mean ± S.D. (triplicates). Values were normalized using actin mRNA expression. Statistical significance among groups is represented by *$P < 0.05$. (B) Time-course analysis of protein levels in 0.5 μM prodigiosin-treated MCF-7 cells subjected to immunobloting with p21 antibody. Vinculin is shown as a loading control and representative blots of independent experiments are shown. (C) [^{3}H]-thymidine incorporation after 24 h-exposure of MCF-7 cells to different doses of prodigiosin. Triplicate experiments were performed and the S.D. is shown. (D) Cell cycle analysis of MCF-7 cells treated with 0.25 and 0.5 μM of prodigiosin.

were analyzed (Fig. 1C). We used low cytotoxic doses of prodigiosin in order to differentiate between cell death and cell cycle blockade. The dose that caused a cell viability decrease of 25% of the cell population (IC_{25} = 0.5 μM) caused 70% ^{3}H-thymidine incorporation, suggesting that this anti-proliferative effect was due to cell cycle blockade. In general, a marked dose-dependent decrease in [^{3}H]-thymidine incorporation was found. Cell cycle progression was analyzed by flow cytometry using propidium iodide in MCF-7 cells exposed to 0.25 and 0.5 μM of prodigiosin for 24 h. Fig. 1D shows a marked accumulation of treated cells in G_0/G_1 (from 66.44% to 84.16% and 82.82%) at both doses exposed. Additionally, the percentage of cells in S phase decreased sharply following prodigiosin treatment thus indicating a significant cell cycle arrest provoked by prodigiosin.

3.2. *p21 induced by prodigiosin is not dependent on p53 accumulation*

The tumor suppressor protein p53 regulates the expression of 21 [23]. To identify whether p53 was responsible for p21 expression after prodigiosin treatment, MCF-7, MCF-7 cells expressing a dominant negative p53 and MDA-MB-231 cells were used. The latter is a human breast cancer cell line that lacks functional p53. Western blot analysis showed that p21 expression correlates with p53 accumulation in the p53 wild-type cell line MCF-7. However, p21 is also induced in MDA-MB-231 cells, while mutated p53 is not accumulated in response to the treatment (Fig. 2A). To further analyze the relationship between p21 and p53, we expressed a dominant negative p53 in MCF-7 cells and analyzed p21 protein levels. We compared p21 levels in MCF-7 cells infected with a dominant negative-expressing retrovirus and cells infected with empty virus as a control. Dominant negative p53 in MCF-7 cells had no effect on p21 expression (Fig. 2B). 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. These results suggest prodigiosin-induced p21 expression is not dependent on p53.

3.3. *Prodigiosin-mediated p21 expression is dependent on TGF-β pathway*

The TGF-β pathway has been reported to induce p21 expression [34]. To determine if prodigiosin induces p21 through a TGF-β dependent mechanism, the levels of p21 mRNA and protein were measured following prodigiosin treatment of MCF-7 cells in the absence or presence of the specific TGF-β receptor type I (TGF-βRI) inhibitor, SB431542 [35]. We observed prodigiosin-induced increase in the amount of p21 mRNA and protein sharply decreased in cells that were pretreated with 20 μM SB431542 (Fig. 3A and B).

Smad-2 is phosphorylated when the TGF-β pathway is activated [17]. The presence of phospho-Smad-2 in non-treated cells (Fig. 3B) indicates that this pathway is already active in MCF-7 cells. This could be due to the fact that they are TGF-β producing cells [36]. The TGF-β receptor smad-dependent pathway remains activated at the onset of

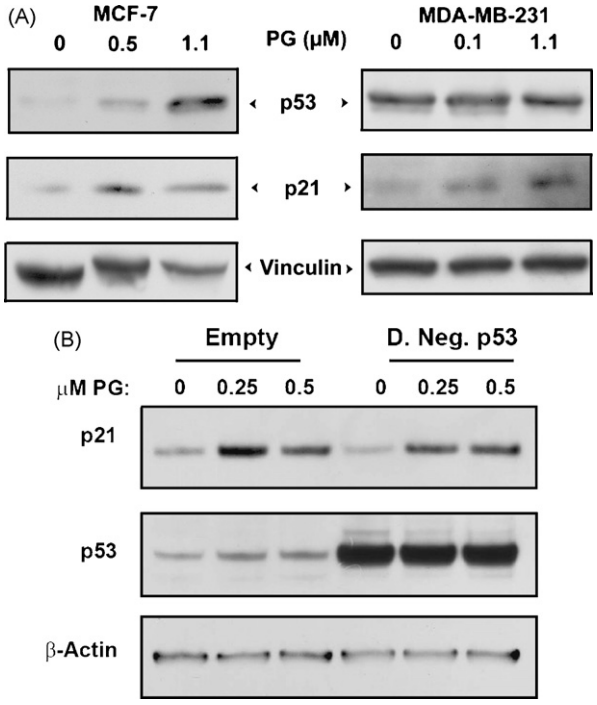

Fig. 2 – Analysis of p21 expression in wild-type p53 and mutated p53 cells after prodigiosin exposure. (A) MCF-7 and MDA-MB-231 cells were incubated with different prodigiosin doses corresponding to their respective IC_{25}, IC_{50} and IC_{75} values at 24 h and then subjected to immunoblot for p53 and p21 detection. Vinculin is shown as a loading control and representative blots of independent experiments are shown. (B) MCF-7 cells were infected with a retrovirus expressing a dominant negative p53 (D.Neg.p53). A pool of infected cells was analyzed for p21 and p53 protein levels after prodigiosin treatment (0.5 μM). β-Actin is shown as a loading control.

prodigiosin treatment. When SB431542 was added phospho-Smad-2 disappeared, indicating that this pathway was inhibited. Therefore, p21 expression seems to depend on the TGF-β pathway. Taken together, these findings suggest that prodigiosin interacts with the TGF-β receptor pathway and TGF-β receptor activity is necessary for p21 induction, as p21 is not expressed when the pathway is inhibited. However, the smad-dependent TGF-β receptor pathway alone is not enough to induce p21 expression, as phospho-Smad-2 is already found in non-treated cells and they do not express p21.

3.4. *Prodigiosin-induced NAG-1 interferes with TGF-β receptor type I*

To further investigate p21 induction, we tried to elucidate which molecule was activating the TGF-β pathway. One possible candidate ligand was NAG-1, a TGF-β family protein that signals through the TGF-β pathway [25]. The gene expression of NAG-1 was up-regulated 79-fold after prodigiosin treatment [15]. Cells were subjected to immunofluorescence after treatment with 0.5 μM of prodigiosin for 24 h, and

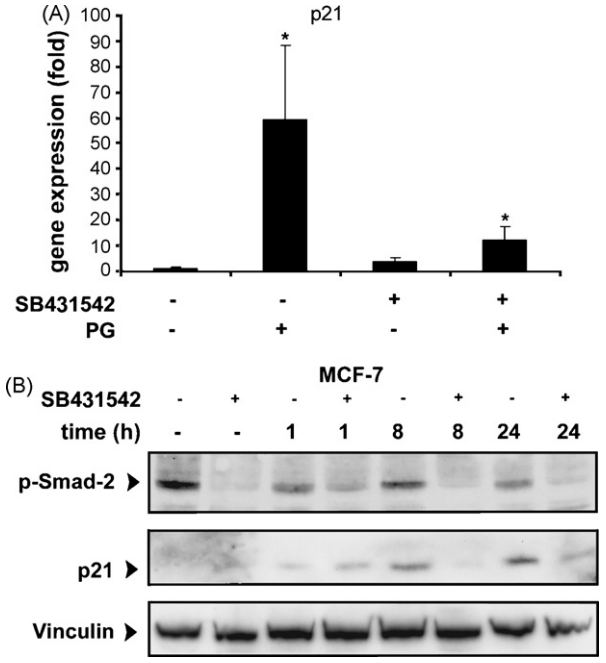

Fig. 3 – p21 regulation by the TGF-β signalling pathway. (A) Cells were incubated with 0.5 μM prodigiosin for 24 h either alone or with 20 μM SB431542. Changes in mRNA levels were analyzed by quantitative real-time RT-PCR. Data were expressed as the mean (columns) ± S.D. (bars) of triplicate experiments and values were normalized using actin mRNA expression (Statistical significance: *P < 0.05). (B) Representative Western blot images of phospho-smad-2, p21 and vinculin (gel loading control) proteins of MCF-7 cells treated for different time periods with 0.5 μM prodigiosin in the absence or presence of 20 μM SB431542.

the NAG-1 protein was seen to accumulate in vesicles throughout the cytoplasm of cells (Fig. 4A, PG). However, cells pre-incubated with 20 μM of the TGF-β pathway inhibitor SB431542 prior to prodigiosin exposure (Fig. 4A, PG + SB431542) showed a similar NAG-1 distribution to those treated solely with prodigiosin. Therefore, inhibition of this pathway does not interfere with prodigiosin-induced NAG-1 expression and cytoplasmic vesicle accumulation. To determine whether prodigiosin-induced NAG-1 could be interacting with the TGF-β pathway, simultaneous incubation with TGF-β receptor type I (TGF-βRI) and NAG-1 antibodies was performed. Co-localization of both proteins in prodigiosin-treated cells was observed, particularly at the membrane surface, suggesting an interaction between NAG-1 and the TGF-β pathway (Fig. 4B). Moreover, since the smad-dependent TGF-β pathway is already activated in MCF-7 cells, we wanted to determine whether prodigiosin had any effect on smad cellular localization. In Figure 4C, we can observe how the majority of smad-2/3 protein in non-treated MCF-7 cells is located in the nucleus. After 4 h of prodigiosin treatment smad-2/3 was still in the nucleus but it was translocated to the cytoplasm after 24 h of treatment. This agrees with our previous results on p-smad protein levels (Fig. 3B).

3.5. *Activation of GSK-3β is required for p21 induction*

GSK-3β is activated by prodigiosin treatment and its activation is necessary for NAG-1 expression induced by prodigiosin [15]. Experiments using a specific inhibitor of this kinase (AR-A014418) were performed. MCF-7 cells were pre-incubated with 50 μM AR-A014418 30 min before treating cells with 0.5 μM prodigiosin for 24 h. We could then observe how p21 gene expression induced by prodigiosin was totally blocked when GSK-3β was inactivated by the inhibitor (Fig. 5A). At the protein level (Fig. 5B), we also observed that p21 accumulation following prodigiosin treatment was blocked by increasing AR-A014418 concentrations. This suggests that GSK-3β activation and p21 expression are dependent. This might be due to GSK-3β induction of NAG-1 expression after prodigiosin treatment, which could lead to TGF-β receptor pathway activation and thus to p21 induction. However, we observed that the AR-A014418 inhibitor induces a dose-dependent increase in p53 protein, which does not induce p21 expression. This corroborates with our previous findings showing that p21 gene induction is independent from p53 protein accumulation (Fig. 2).

3.6. *The TGF-β pathway is not implicated in prodigiosin-induced apoptosis*

Cell viability experiments were performed in order to find out whether the TGF-β pathway also contributes to the apoptotic phenotype induced by prodigiosin (Fig. 6A). MCF-7 cells were pre-treated with 20 μM SB431542. An apoptotic concentration of prodigiosin (IC_{75}) was then added. A 70% decrease in cell viability was observed in MCF-7 cells treated with prodigiosin. No recovery in cell viability was observed when pre-treating cells with 20 μM SB431542. Hence, the TGF-β pathway is not involved in prodigiosin-induced apoptosis. Moreover, death receptor proteins 4 and 5 have been related to NAG-1 overexpression [37]. Prodigiosin treatment also up-regulates the expression of these death receptor proteins [15]. Gene expression quantification experiments were performed to analyze whether the expression of death receptors could be regulated through the TGF-β pathway. Fig. 6B shows a significant increase in DR-4 and DR-5 mRNAs (5- and 14-fold higher than the control, respectively) after 24 h-prodigiosin treatment. No significant modifications occurred when adding 20 μM SB431542 (3 and 11).

4. Discussion

The negative cell-cycle regulator p21 has previously been reported as one of the most significantly up-regulated genes in breast cancer cells after treatment with the anticancer drug prodigiosin [15]. The aim of the present study was to identify the molecular mechanisms that triggered prodigiosin-induced p21 expression. Here we demonstrate that p21 expression is independent of the tumor suppressor protein p53, but dependent on the activation of the TGF-β signaling pathway and on GSK-3β kinase activity. We also suggest that this pathway might be activated by the interaction between the cytokine NAG-1 and the TGF-β pathway receptors. Finally, we

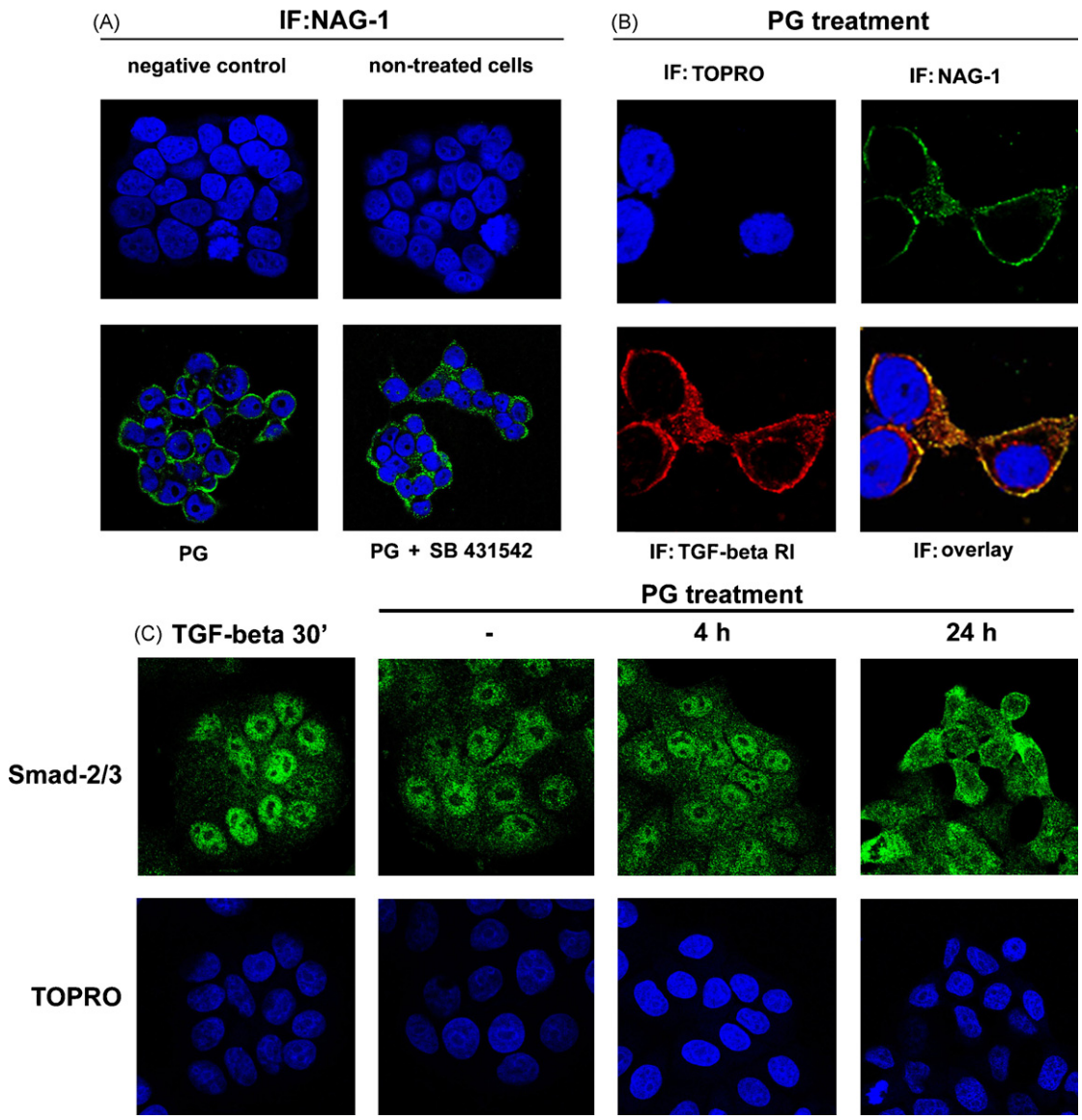

Fig. 4 – Cellular localization of NAG-1, TGF-βRI and smad-2/3. (A) Immunocytochemistry for NAG-1 detection was performed in MCF-7 cells ("negative control" [incubated without NAG-1 antibody] and "non-treated cells") and in cells treated with 0.5 μM prodigiosin for 24 h in the absence ("PG") or presence of 20 μM SB431542 ("PG + SB431542"). (B) MCF-7 cells exposed to 0.5 μM prodigiosin for 24 h were incubated with NAG-1 and TGF-βRI antibodies simultaneously; nuclear staining with TO-PRO®-3 iodide (TOPRO) was also performed. Representative immunofluorescent images from three independent experiments are shown. (C) MCF-7 cells were exposed to 0.5 μM prodigiosin for different time periods and then were incubated with smad-2/3 antibody; nuclear staining with TO-PRO®-3 iodide (TOPRO) was also performed. Representative immunofluorescent images from three independent experiments are shown.

observe that this pathway is not involved in prodigiosin cytotoxicity, although it might contribute to prodigiosin's cytostatic properties.

p21 is a cyclin-dependent kinase (CDK) inhibitor that belongs to the Cip/Kip family of CDK inhibitors. Cell cycle progression is blocked when the catalytic activity of (CDK)-cyclin complexes is inhibited by the binding of a CDK inhibitor molecule, such as p21 [22]. Cells exposed to stress signals, such as DNA-damaging agents, induce p21 expression, which leads to cell cycle arrest [23]. It has also been shown that overexpression of p21 results in G_1 and G_2 arrest [38]. Here we observe that prodigiosin treatment provokes cell cycle arrest as well as p21 induction. Likewise, the blockade of cell-cycle progression in response to prodigiosin was previously described in hematopoietic cancer cells [12]. Altogether, these results suggest that p21 might be involved in cell cycle arrest induced by prodigiosin treatment.

p21 gene expression can be transcriptionally regulated by a wide variety of different molecules [24]. It has been extensively described that p21 is a target gene of the tumor suppresor protein p53 [39]. After prodigiosin treatment, p21 gene expression was more than 34-fold higher than in non-treated

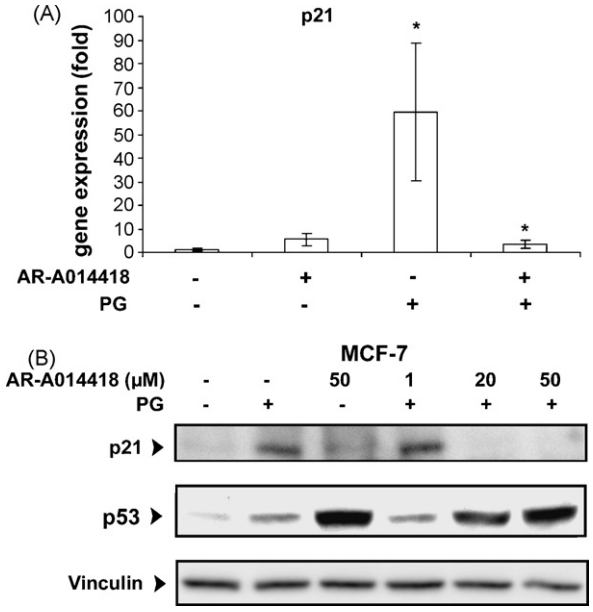

Fig. 5 – p21 regulation by GSK-3β. (A) MCF-7 cells were exposed to 0.5 μM prodigiosin for 24 h in the absence or presence of 50 μM AR-A014418 and changes in gene expression (fold changes with respect to control cells) were evaluated by quantitative real time RT-PCR. Columns are expressed as the means of three independent experiments, bars are S.D. Significant ($P < 0.05$) induction by prodigiosin or inhibition when combined with AR-A014418 is indicated by *. (B) After treating cells with 0.5 μM prodigiosin for 24 h with or without 1, 20 or 50 μM AR-A014418, cell lysates were collected for Western blot analysis using p21, p53 and vinculin antibodies. The latter is shown as a gel loading control. Representative blots from independent experiments are shown.

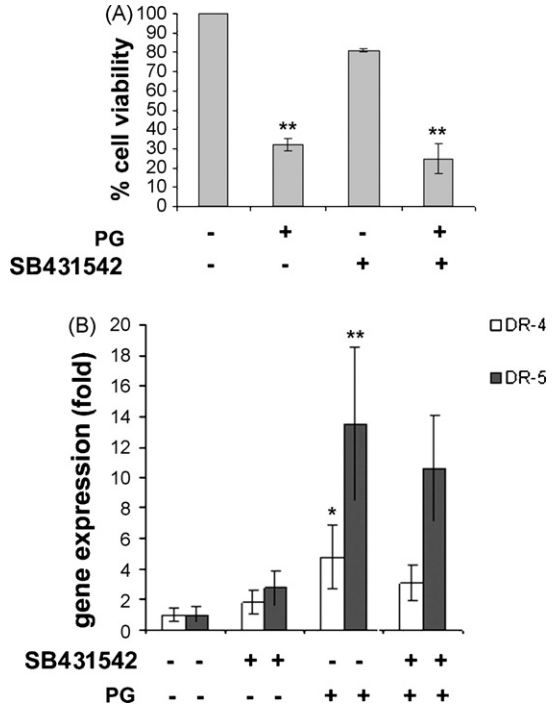

Fig. 6 – Analysis of the role of the TGF-β pathway in prodigiosin-induced apoptosis. (A) Cells were incubated with 1.4 μM prodigiosin for 24 h alone or in the presence of 20 μM SB431542 and cell viability was measured by the MTT assay. Data are expressed as the percentage of non-treated cells and shown as the mean (triplicate experiments) ± S.D. and statistical significance is indicated by *$P < 0.05$; **$P < 0.01$. (B) mRNA from MCF-7 cells that were either not treated or treated with 20 μM SB431542 (prior to 0.5 μM prodigiosin treatment for 24 h) was extracted and DR-4 and DR-5 levels were quantified by quantitative real-time RT-PCR. Data are presented as the mean of triplicate experiments (columns) ± S.D. (bars). Statistical significance is indicated by *$P < 0.05$; **$P < 0.01$.

cells. We observed that p21 gene expression was induced in a p53-independent way. This might represent an advantage in the clinical treatment of tumors, as the p53 protein is mutated in most human tumors. This mutation prevents cancer cells from suffering the cytostatic and/or cytotoxic effects of anticancer drugs [40].

The TGF-β pathway can also induce p21 expression [41] and this pathway is already activated in MCF-7 cells. The cytostatic and apoptotic functions of this pathway help control the homeostasis of normal tissues. The loss of these effects leads to hyperproliferative disorders [18]. Late stage human carcinomas, especially advanced breast cancers [42], often become resistant to TGF-β growth inhibition. They also overproduce this cytokine, probably to create a local immunosuppressive environment that promotes tumor growth and intensifies the invasive and metastatic behaviour of the tumor cells themselves [43]. Both features have been described in MCF-7 cells [36,42] and might explain why MCF-7 cells continue proliferating even when the TGF-β pathway is already active. The Smad-dependent pathway has previously been related to pro-metastatic properties and tumor cell invasiveness [44], hence this might be one advantage that its continuous activation may give to MCF-7 cells.

TGF-β pathway activation mediated by a TGF-β type I receptor has been shown to be necessary for p21 induction after prodigiosin treatment, but smad phosphorylation and translocation to the nucleus are not enough to induce p21 expression, as shown in MCF-7 cells. Other molecular pathways, which are or are not dependent on TGF-β pathway activation, also seem to interact with the p21 promoter. TGF-β family members also signal through a smad-independent TGF-β pathway. These include as downstream effectors the extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), p38 MAPK, phosphatidylinositol-3 kinase (PI3K), TGF-β-activated kinase 1 (TAK1), protein phosphatase 2A (PP2A) and Rho GTPases [20]. In particular, it has been shown that the TGF-β family member NAG-1 activates the smad-dependent TGF-β pathway [25] but also some MAPK signaling pathways [45]. In addition, after prodigiosin cell exposure, NAG-1 is over expressed [15] and colocalizes with the TGF-β type I receptor. Therefore, it might bind to its receptor and activate some other molecule that is different than smads. This molecule, in collaboration or not with

smads, may be responsible for p21 induction after prodigiosin treatment. In this regard, prodigiosin is known to activate the p38 MAPK kinase after 15 min of treatment in jurkat cells [46]. The p38 MAPK classes of protein kinases are activated by stress signals and mediate cellular responses, including steps in the apoptosis and maturation of some cell types [47]. Therefore, one possibility is that NAG-1 may bind to the TGF-β receptor, which in turn activates p38 inducing p21 expression through the sp1 transcription factor. This mechanism of action has previously been described in other compounds, such as benzyl isothiocyanate [48]. Besides this, NAG-1 is also capable of inducing cell-cycle arrest through p21 induction in ovarian cancer cells [49]. This fits with our proposed mode of prodigiosin-induced cell cycle arrest via the TGF-β pathway activated by the cytokine NAG-1.

However, an additional molecular pathway necessary for p21 induction could be independent of the TGF-β pathway. Prodigiosin provokes GSK-3β activation through AKT dephosphorylation [15]. This kinase is a negative regulator of p21 expression. The negative regulation occurs by exporting a transcription factor, called FoxO, necessary for TGF-β-stimulated p21 promoter activation [50] to the cytoplasm. Therefore, prodigiosin could be inducing AKT dephosphorylation, thus enabling FoxO to collaborate with the smads that still remain in the nucleus at early times of prodigiosin treatment in order to induce p21 expression.

In conclusion, among the molecular mechanisms of action of the anticancer agent prodigiosin, induction of the p21 cell cycle inhibitor through activation of the TGF-β pathway has been observed. This process might lead to cell-cycle arrest but it is not involved in the cytotoxic properties of this molecule. Altogether, these results shed light on the molecular mechanism of the action of prodigiosin and might explain its well-documented pharmacological effects as an immunosuppressor.

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