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Small-Molecule Prodigiosin Restores p53 Tumor Suppressor Activity in Chemoresistant Colorectal Cancer Stem Cells via c-Jun-Mediated ΔNp73 Inhibition and p73 Activation

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Abstract

Tumor suppressor p53 is frequently mutated or inactivated in colorectal cancer. In contrast, p53 family member p73 is rarely mutated in colorectal cancer and p73 activation elicits p53-like tumor suppression. Colorectal cancer stem cells (CRCSC) comprise a rare self-renewing subpopulation that contributes to tumor maintenance and chemoresistance. p53 restoration is known to target CRCSCs, but p73 restoration in CRCSCs has not been examined. In this study, we investigated the effects of the small-molecule prodigiosin, which restores the p53 pathway in tumor cells via p73 activation, on CRCSCs *in vitro* and *in vivo*. Prodigiosin prevented colonosphere formation independent of p53 status and reduced the viability of self-renewing, 5-fluorouracil-resistant Aldefluor positive [Aldefluor(+)] CRCSCs *in vitro*. Furthermore, prodigiosin inhibited the growth of xenograft tumors initiated with Aldefluor+ cells without toxic effects and limited the tumorigenic potential of these cells. Consistently, prodigiosin induced activation of a p53-responsive luciferase reporter in colonospheres, Aldefluor(+) cells, and tumor xenografts. Mechanistic studies revealed that prodigiosin increased the levels of p73 and reduced levels of the oncogenic N-terminally truncated isoform ΔNp73 in Aldefluor(+) cells. Accordingly, p73 knockdown or ΔNp73 overexpression suppressed prodigiosin-mediated inhibition of colonosphere formation. Moreover, prodigiosin increased levels of the transcription factor c-Jun, a regulator of p73 and ΔNp73, in both the cytoplasm and nucleus. c-Jun knockdown attenuated prodigiosin-mediated p53-reporter activation, ΔNp73 downregulation, p73 activation, and cell death. Collectively, our findings highlight the previously uncharacterized use of p73-activating therapeutics to target CRCSCs. *Cancer Res; 76(7); 1989–99. ©2016 AACR.*

Introduction

Despite the approval of targeted molecular agents for therapy, more than 50,000 people are expected to die of colorectal cancer in the United States in 2014 (1). Inactivation of the p53 tumor suppressor is a key event in colorectal cancer and correlates with the transition from benign adenoma to malignant carcinoma (2). p53 elicits its tumor suppressive effects through target genes that mediate cell-cycle arrest, senescence, or apoptosis in response to various cellular stresses (3). Loss of p53 function via mutation or allelic loss also contributes to resistance to chemotherapy (2, 4). p53 pathway restoration is a potentially safe and potent approach for anticancer therapy (3).

p73 is a member of the p53 family of transcription factors and has a high degree of homology in the DNA-binding domain with p53 (5). p53 and p73 share several common target genes, however, p73 is also known to activate p53-independent target genes (6). In contrast to p53, p73 is rarely mutated in colorectal cancer (7). In addition, p73 restoration elicits a p53-like tumor suppressive effect. Thus, p73 restoration is an attractive alternative approach for p53 pathway restoration in tumors with wild-type and mutant p53. p73-activating small molecules are potent antitumor agents *in vitro* and *in vivo* (8–10). The p73 gene undergoes alternative mRNA splicing generating various isoforms with opposing effects. The oncogenic N-terminally truncated isoform ΔNp73 is a dominant negative inhibitor of p73 and p53 (11–13). ΔNp73 levels correlate with poor overall survival in colorectal cancer patients (14). p73 transcriptional activity and tumor suppression is also blocked by mutant p53 binding (15, 16). Clearly, it is important to identify approaches that activate p73-mediated tumor suppression and also prevent oncogenic mechanisms that block p73 activity. c-Jun, a member of the AP-1 family of transcription factors is known to regulate the induction of p73 and

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degradation of ΔNp73 in response to cellular stress (17, 18). Thus, c-Jun activation maybe an attractive approach for p53 pathway restoration via p73.

Colorectal cancer is driven by a rare subpopulation of colorectal cancer–initiating stem cell–like cells (CRCSC) capable of long-term self-renewal, colorectal cancer initiation, and maintenance (19, 20). CRCSCs are responsible for colorectal cancer chemotherapy resistance (21, 22), recurrence (23), and metastasis (22, 24). Clearly, CRCSCs are an important therapeutic target in colorectal cancer. Interestingly, non-CRCSC bulk tumor cells can also dedifferentiate to give rise to CRCSCs (25, 26). Thus, colorectal cancer therapy must incorporate approaches to target both bulk tumor cells and CRCSCs.

Recent evidence suggests that regulation of stem cell self-renewal and differentiation is an important aspect of the tumor suppressive role of p53. Loss of p53 function correlates with poor tumor differentiation and enrichment of the stem cell phenotype (27). Small-molecule–mediated p53 pathway restoration has been shown to target the tumor stem cell population (28, 29). p73 is an important regulator of neural stem cell maintenance and differentiation (30–32). Induced pluripotent cell generation efficiency is improved by ΔNp73 (33). Low expression of p73 correlated with cisplatin resistance of glioblastoma stem cells and p73 overexpression increased cisplatin-mediated apoptosis in glioblastoma stem cells (34). However, the effects of p73 restoring small molecules on tumor stem cells remain unknown.

In a p53-responsive luciferase reporter–based high-throughput screen in mutant p53 expressing colorectal cancer cells (8), we recently identified small-molecule prodigiosin as a potent p53 pathway restoring agent. Prodigiosin-mediated p53 pathway restoration *in vitro* and *in vivo* involved p73 activation and disruption of the mutant p53–p73 interaction (35). We hypothesized that prodigiosin-mediated p53 pathway restoration via p73 targets both bulk tumor cells and therapy-resistant CRCSCs. In the current study, we explored whether the potent antitumor effects of prodigiosin in colorectal cancer involve targeting of CRCSC self-renewal. Prodigiosin prevented the self-renewal of 5-fluorouracil (5-FU)–resistant CRCSCs *in vitro* and *in vivo*. Mechanistically, prodigiosin-mediated targeting of CRCSCs involved p53 pathway restoration via c-Jun-mediated activation of p73 and inhibition of ΔNp73.

Materials and Methods

Cell culture and reagents

All colorectal cancer cell lines were obtained from the ATCC. SW480, DLD1, and HCT116 cells expressing a p53-responsive luciferase reporter were generated in our laboratory in 2003 (8). SW480 and DLD1 p53 reporter cells with stable p73 knockdown were previously established in our laboratory in 2012 to 2013 (35, 36). Cells were authenticated every month by bioluminescence, growth, and morphologic observation. Cells in adherent culture were maintained in McCoy 5A or DMEM containing FBS and penicillin–streptomycin (Invitrogen) at 37°C in 5% CO_2. Prodigiosin was obtained from the NCI/DTP Open Chemical Repository and 5-FU was ordered from Sigma. Dimethyl sulfoxide (DMSO, Fisher) was used as the vehicle. c-Jun siRNA was obtained from Santa Cruz Biotechnology and ΔNp73 overexpression plasmid was obtained from OriGene. Transient plasmid transfection was performed with Lipofectamine 2000 and siRNA transfection was performed with Lipofectamine RNAiMAX (Invitrogen) as described previously (37). Nuclear-cytoplasmic fractionation was performed with the NE-PER Nuclear and Cytoplasmic Extraction Reagent (Life Technologies).

Aldefluor assay

The Aldefluor assay was performed according to the manufacturer's protocol (ALDEFLUOR Kit, STEMCELL Technologies). Cells resuspended with Aldefluor diethylaminobenzaldehyde (DEAB) reagent, an inhibitor of Aldefluor activity, served as a negative control. Aldefluor positive cells [Aldefluor(+)] and Aldefluor negative cells [Aldefluor(−)] were detected by flow cytometry as described previously (37).

FACS

FACS sorting was performed with the Becton Dickinson FACSAria SORP Cell Sorter at the Penn State Hershey flow cytometry core. Aldefluor(+) and Aldefluor(−) cells were gated as per the DEAB negative control and sorted cells (99% purity) were collected in media. Aldefluor(+) cells were maintained under nonadherent conditions and Aldefluor(−) cells were grown in adherent culture (37).

Colonosphere culture

Colonospheres were grown under nonadherent growth conditions using the MammoCult Human Medium (STEMCELL Technologies) in ultra-low attachment plates (Corning) as per the manufacturer's protocol. Cells (1,000–20,000 per well) were seeded in MammoCult medium containing DMSO or prodigiosin. Colonospheres of size > 60 μm were counted and imaged 3 to 7 days after seeding. Medium was changed every 3 to 7 days to maintain Aldefluor(+) cells in culture (37).

Holoclone assay, cell viability, and sub-G_1 analysis

For the holoclone assay, cells treated with DMSO or prodigiosin were counted using Trypan blue and viable cells were passaged at various clonal densities (50–5,000 cells). Colony formation was determined by crystal violet staining upon passage (38). Cell viability was determined using the CellTiter-Glo reagent (Promega) as per the manufacturer's protocol and bioluminescence imaging with IVIS imaging system (Xenogen; ref. 35). For sub-G_1 analysis, cells were treated, trypsinized, ethanol-fixed, stained with propidium iodide (Sigma), and analyzed by flow cytometry as described previously (8).

Xenograft and passage studies

All animal experiments were performed as per approved protocols of the Institutional Animal Care and Use Committee at the Penn State Hershey Medical Center. Athymic nu/nu mice (Charles River Laboratories, 4- to 6-week-old female mice) were used for subcutaneous xenografts. Cells (1–2 million cells per injection) were subcutaneously injected as a suspension [1:1 in Matrigel (BD Biosciences) and PBS] into the right and left flank of the mice. Prodigiosin treatment was started when the tumors reached a detectable volume (~125 mm^3). Mice were administered three doses of DMSO or prodigiosin (5 mg/kg, i.p.) at 5- or 7-day intervals. Tumor growth and body weight was monitored until the endpoint and tumor volume was calculated as $((\text{length} + \text{width})/2)^3$.

For the passage experiment, harvested tumors were digested with Collagenase type 3 (Worthington, 155 units/mL) in sterile

Table 1. Prodigiosin prevents *in vivo* passage of Aldefluor(+) CRCSC-initiated tumors

Sample	Tumor formation upon passage
Control	6/6
Prodigiosin	2/6

NOTE: Single cells from DLD1 Aldefluor(+) xenograft tumors treated with DMSO or prodigiosin (Fig. 3B) were reinjected into athymic nude mice to determine tumor formation. One million viable cells were used for each passage injection. Tumor formation was determined at day 20 after tumor implantation.

RPMI (Mediatech, Inc., serum- and antibiotic-free). After digestion, tumor cells were passed through a 100-μm filter and reinjected into mice as described above (1 million viable cells).

Hematoxylin and eosin staining and IHC

Hematoxylin and eosin staining (H&E) was performed as described previously (35). H&E staining of paraffin-embedded tumor and tissue sections was performed at the Penn State Hershey Medical Center Histology Core Facility. IHC for CD44 (Cell Signaling Technology) was performed as described previously (37).

Serum chemistry analysis

Serum chemistry analysis for electrolytes, alkaline phosphatase, creatinine, albumin, and blood urea nitrogen was performed at the Penn State Hershey Comparative Medicine Diagnostic Laboratory. Mice were administered two doses of DMSO or prodigiosin (5 mg/kg, i.p.) at a 3-day interval. Blood was harvested by terminal cardiac puncture from anesthetized mice. Blood was allowed to clot at room temperature, centrifuged, and the serum obtained was submitted for analysis.

p53-responsive luciferase reporter assay

Bioluminescence imaging of the p53-responsive luciferase reporter–expressing cells was performed *in vitro* and *in vivo* as described previously (8, 35). The IVIS imaging system (Xenogen) was used to detect luciferase activity. Imaging was performed 4 to 12 hours after prodigiosin treatment of cells or mice with xenograft tumors. Prior to imaging, cells or mice were treated with D-luciferin intraperitoneally (Gold Biotechnology). Imaging was performed 5 minutes following treatment with D-luciferin.

Western blot analysis

Western blotting was performed as described previously (35). The following antibodies were used: p73 (Imgenex and Bethyl), ΔNp73 (Imgenex), c-Jun (BD), phospho c-Jun (Cell Signaling Technology), GAPDH (Sigma), Lamin (Cell Signaling Technology) p21 (Calbiochem), Jagged-1 (Cell Signaling Technology), cleaved caspase-8 (Cell Signaling Technology), cleaved PARP (Cell Signaling Technology), β-actin (Sigma), CD44 (Cell Signaling Technology), ALDH (BD), ID1 (Santa Cruz Biotechnology), ID3 (Santa Cruz Biotechnology), E-cadherin (Cell Signaling Technology), and Ran (BD Biosciences).

Statistical analysis

Data are presented as the mean ± SD or SE of mean from at least three replicates. The Student two-tailed *t* test in Excel (Microsoft) was used for pairwise analysis. Statistically significant changes (*) are indicated in the figures with *P* values.

Results

Prodigiosin reduces colonosphere, holoclone formation of multiple colorectal cancer cell lines, and downregulates CRCSC markers

Colonosphere culture enriches for CRCSC markers compared with adherent culture and represents a functional *in vitro* model for studying colorectal cancer self-renewal (39). We observed that colonosphere culture enriches for Aldefluor(+) CRCSCs compared with adherent culture (Supplementary Fig. S1A). To determine whether prodigiosin targets colorectal cancer self-renewal *in vitro*, we tested the effects of prodigiosin on colonosphere formation. Prodigiosin significantly reduced colonosphere formation of SW480, SW620, HCT116, and DLD1 cells in a dose-dependent manner (Fig. 1A). In DLD1 cells, colonospheres that formed in the presence of prodigiosin were smaller in size as compared with DMSO (Fig. 1B). Also, colonospheres formed in the presence of prodigiosin were smaller than those formed with first line colorectal cancer chemotherapeutic agent 5-FU (Fig. 1C; ref. 40). The doses of 5-FU and prodigiosin tested were comparable in terms of their effects on cell viability (Supplementary Fig. S1B). Sub-G_1 analysis indicated that prodigiosin-induced cell death in cells grown as colonospheres in a dose-dependent manner (Fig. 1D). Holoclone formation is a clonogenic assay that has been used to study cancer stem cell properties (38). To further test the effects of prodigiosin on colorectal cancer self-renewal, we examined holoclone formation upon prodigiosin treatment. DLD1 and RKO cells were treated with DMSO or prodigiosin and viable cells were replated at various clonogenic densities. Prodigiosin-reduced holoclone formation in both colorectal cancer cell lines tested (Supplementary Fig. S1C). We also tested the effects of prodigiosin on the CRCSC markers ALDH (41), CD44 (42), ID1, ID3 (43), and epithelial-to-mesenchymal transition marker E-Cadherin (44). ALDH, CD44, ID3, and E-cadherin levels were reduced in colonospheres formed upon *in vitro* passage of DLD1 cells treated with prodigiosin (Fig. 1E). Thus, prodigiosin targets the self-renewal properties of colorectal cancer cells *in vitro*.

Prodigiosin decreases colonosphere formation, downregulates self-renewal markers, and reduces the viability of 5-FU–resistant Aldefluor(+) CRCSCs

To test the effects of prodigiosin specifically on CRCSCs we used a marker-based approach by sorting for Aldefluor(+) cells (41). Postsort validation showed that FACS sorting highly enriched for Aldefluor(+) cells compared with unsorted cells (Supplementary Fig. S1D). Prodigiosin significantly reduced colonosphere formation of HCT116 and SW480 Aldefluor(+) cells in a dose-dependent manner (Fig. 2A and Supplementary Fig. S1F). 5-FU could not reduce colonosphere formation of Aldefluor(+) cells whereas prodigiosin decreased colonosphere formation at equivalent doses in terms of effects on cell viability (Fig. 2C and Supplementary Fig. S1B). In the experiments described in Fig. 1 and 2, colonosphere formation was assessed in medium containing prodigiosin for 3 to 6 days. To determine whether prodigiosin can reduce colonosphere formation with shorter treatments, cells were treated for 12 and 24 hours with prodigiosin and examined for colonosphere formation. Prodigiosin significantly decreased colonosphere formation even with the shorter treatments tested (Supplementary Fig. S1E). CD44, ID1, ID3, and E-cadherin levels

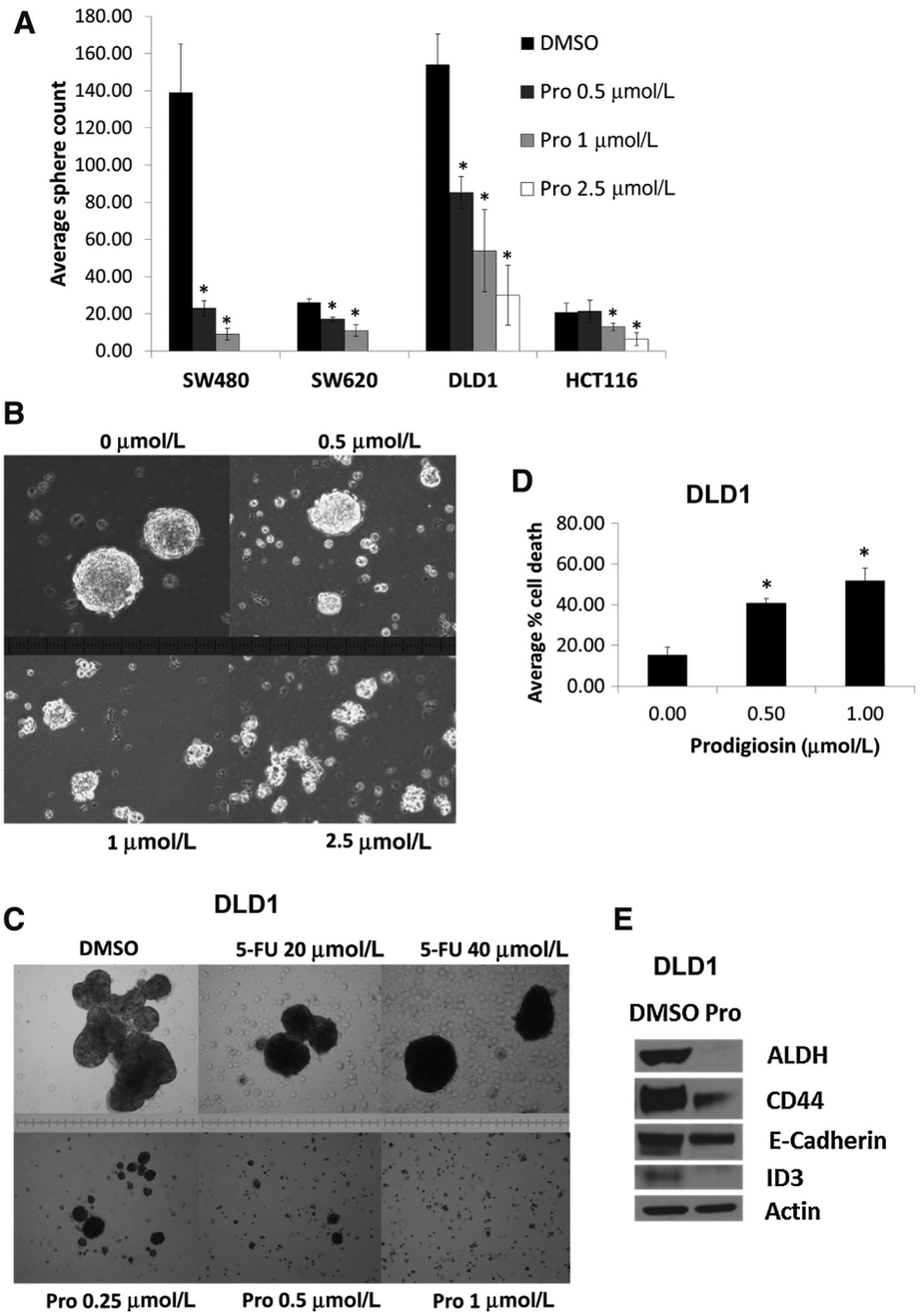

Figure 1.
Prodigiosin reduces colonosphere formation and downregulates self-renewal markers. A, SW480, SW620, HCT116, and DLD1 cells were plated for colonosphere formation in the presence of DMSO or 0.5/1/2.5 μmol/L prodigiosin (Pro). Colonospheres (> 60 μm) were counted after 3 to 4 days. *, $P < 0.05$. B, representative image (magnification, ×20) for DLD1 cells in A. Each division on the scale is 10 μm. C, DLD1 cells were plated for colonosphere formation in the presence of DMSO or 20/40 μmol/L 5-FU or 0.25/0.5/1 μmol/L prodigiosin and imaged (magnification, ×10) after 6 days. Each division on the scale is 10 μm. D, DLD1 cells were plated for colonosphere formation in the presence of DMSO or 0.5/1 μmol/L prodigiosin, harvested after 4 days, and cell death was determined by sub-G_1 analysis. *, $P < 0.02$. E, DLD1 cells were treated with DMSO or 1 μmol/L prodigiosin for 24 hours, viable cells were replated in colonosphere culture for 24 hours, and Western blot analysis was performed for ALDH, CD44, ID3, E-cadherin, and actin.

were reduced upon prodigiosin treatment of Aldefluor(+) cells but not with 5-FU treatment (Fig. 2B). Thus, prodigiosin targets CRCSC self-renewal *in vitro*.

Cell viability analysis was performed to compare the effects of prodigiosin and 5-FU on CRCSCs and non-CRCSCs. Prodigiosin reduced the viability of 5-FU–resistant Aldefluor(+) CRCSCs in a dose-dependent manner (Fig. 2D). Interestingly, prodigiosin reduced the viability of both Aldefluor(+) and unsorted cells whereas 5-FU reduced the viability of only unsorted cells (Fig. 2E). Thus, chemotherapy can target only bulk tumor cells, whereas prodigiosin targets both CRCSCs and bulk tumor cells.

Prodigiosin prevents Aldefluor(+) CRCSC-initiated xenograft tumor growth and passage without toxic effects

We tested the effects of prodigiosin on CRCSCs *in vivo* using a CRCSC-initiated xenograft tumor model. Athymic nude mice with xenograft tumors initiated with Aldefluor(+) cells were treated with three doses of prodigiosin every 5 to 7 days. The optimal *in vivo* dose of prodigiosin for antitumor effects was determined previously (35). Prodigiosin significantly reduced SW480 and DLD1 Aldefluor(+) CRCSC-initiated xenograft tumor growth (Fig. 3A and B, Supplementary Fig. S2A and S2B). Tumor growth did not resume even in the absence of

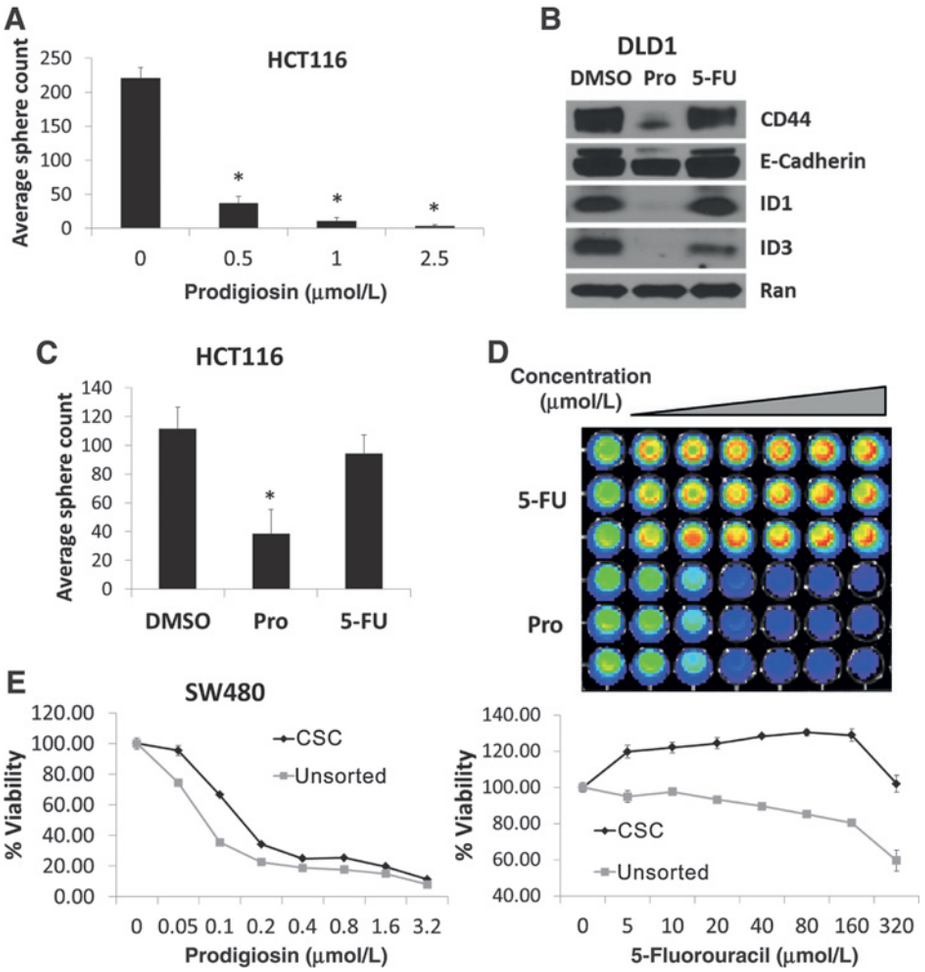

Figure 2.
Prodigiosin decreases colonosphere formation, downregulates CRCSC markers, and reduces the viability of 5-FU–resistant Aldefluor(+) CRCSCs. A, HCT116 Aldefluor(+) cells were plated for colonosphere formation in the presence of DMSO or indicated doses of prodigiosin (Pro). Colonospheres (> 60 μm) were counted after 3 to 6 days. *, $P < 0.006$. B, DLD1 Aldefluor(+) cells were plated in colonosphere culture in the presence of DMSO or 1 μmol/L prodigiosin or 100 μmol/L 5-FU for 24 hours. Western blot analysis was performed for CD44, E-cadherin, ID1, ID3, and Ran. C, HCT116 Aldefluor(+) cells were plated for colonosphere formation in the presence of DMSO or 0.5 μmol/L prodigiosin or 40 μmol/L 5-FU for 6 days. *, $P < 0.05$. D, SW480 Aldefluor(+) cells were plated and treated with DMSO or increasing doses of prodigiosin (0.05-3.2 μmol/L) or 5-FU (5-320 μmol/L) for 60 hours and cell viability was determined. E, sorted SW480 Aldefluor(+) and unsorted cells were plated, treated with DMSO or indicated doses of prodigiosin or 5-FU for 60 hours, and cell viability was determined.

continued prodigiosin treatment, indicating an effect on self-renewing CRCSCs (Fig. 3A).

To validate the CRCSC marker used in our study, we performed an *in vivo* passage assay of xenograft tumor formation with Aldefluor(+) and Aldefluor(−) cells. Upon initial injection of a million cells both Aldefluor(+) and Aldefluor(−) cells formed subcutaneous xenograft tumors in athymic nude mice (data not shown). However, upon passage of viable cells from the initial tumors into mice, we observed that only Aldefluor(+) cells formed tumors (Fig. 3C). Thus, Aldefluor(+) cells represent the CRCSC population *in vivo*.

Next, we tested whether prodigiosin could prevent Aldefluor (+) CRCSC *in vivo* passage. Viable cells from DMSO and prodigiosin-treated DLD1 Aldefluor(+) CRCSC-initiated tumors were reinjected into mice (in the absence of DMSO or prodigiosin treatment). DMSO-treated tumor cells formed six tumors in six injections whereas prodigiosin-treated tumor cells formed two tumors in six injections (Table 1). The tumors that formed with prodigiosin-treated cells were smaller than those formed with DMSO-treated cells (Supplementary Fig. S2C). Also, the tumor cell density was decreased in tumors that formed with prodigiosin-treated cells compared with tumors formed with DMSO-treated cells (Fig. 3D). CD44 expression was reduced in prodigiosin-treated passaged tumors compared with DMSO treatment (Supplementary Fig. S2D). Thus, prodigiosin targets CRCSC self-renewal *in vivo*.

To determine the safety profile of prodigiosin in mice, we monitored body weight, performed serum chemistry analysis, and examined normal tissue histology. Prodigiosin treatment did not result in significant changes in the body weight of athymic nude mice (Supplementary Fig. S3A and S3B). We did not observe any changes in liver, kidney, heart, spleen, lung, and gastrointestinal tract histology in prodigiosin-treated mice compared with DMSO-treated mice (Fig. 3E). Serum chemistry analysis for selected markers such as electrolytes (sodium, potassium, and chloride), alkaline phosphatase, creatinine, albumin, and blood urea nitrogen revealed no significant changes except a significant decrease in alkaline phosphatase upon prodigiosin treatment (Supplementary Fig. S3C). Thus, we observed an apparent lack of toxicity in mice treated with the therapeutic dose of prodigiosin.

Prodigiosin restores the p53 pathway in CRCSCs *in vitro* and *in vivo*

To determine the mechanism of prodigiosin-mediated anti-CRCSC effects, we examined whether prodigiosin can restore the

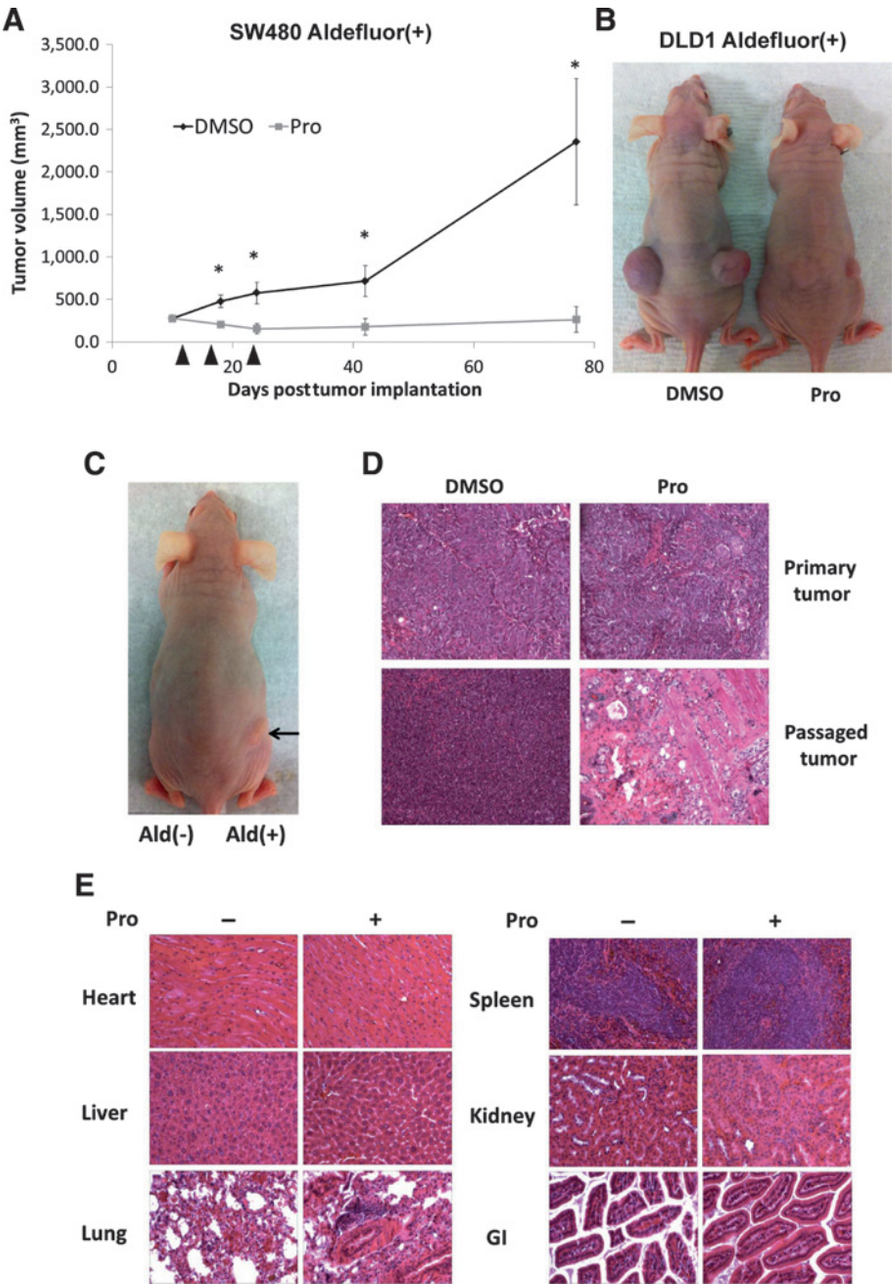

Figure 3.
Prodigiosin prevents Aldefluor(+) CRCSC-initiated xenograft tumor growth and passage without toxic effects. A, SW480 Aldefluor(+) cells were injected subcutaneously into the right and left flanks of athymic nude mice. Mice were administered either DMSO or prodigiosin (Pro) 5 mg/kg (i.p.; $n = 5$ mice per group) upon tumor formation. Doses (indicated by triangle) were administered posttumor implantation on day 11, 16, and 22. *, $P < 0.05$. B, DLD1 Aldefluor(+) cells were injected subcutaneously into the flanks of athymic nude mice. Upon tumor formation, mice were administered DMSO or prodigiosin 5 mg/kg (i.p.; $n = 5$ mice per group) on day 7, 14, and 21 posttumor implantation. Representative image is shown. C, sorted DLD1 Aldefluor(+) [Ald (+)] and Aldefluor(−) [Ald(−)] cells (1 million cells per injection) were subcutaneously injected into the right and left flank, respectively, of athymic nude mice. Viable cells from initial tumors were reinjected into mice to determine tumor formation at day 20. Representative image for tumor formation upon passage is shown. D, H&E staining of initial tumors from B and passaged tumors from Table 1. E, H&E staining of liver, kidney, heart, spleen, lung, and gastrointestinal tract (GI) from athymic nude mice treated with two doses of DMSO or prodigiosin (5 mg/kg) at a 3-day interval. Tissues were obtained 3 days after the second dose.

p53 pathway in CRCSCs. We have previously demonstrated that prodigiosin restores the p53 pathway in colorectal cancer cells (35). Using p53-responsive luciferase reporter–expressing colorectal cancer cells (8), we determined prodigiosin-mediated effects on p53 pathway transcription in colonospheres, Aldefluor(+) cells and Aldefluor(+) CRCSC-initiated xenograft tumors. Prodigiosin induced p53-responsive luciferase reporter activity in DLD1 colonospheres (Fig. 4A and Supplementary Fig. S4A) at various doses. The observed increase in reporter activity was not due to a difference in the number of colonospheres (Supplementary Fig. S4A). Prodigiosin significantly restored p53 pathway transcription in SW480 Aldefluor(+) cells *in vitro* at various doses and time points tested (Fig. 4B and C). We also observed prodigiosin-mediated p53 pathway restoration *in vivo* in xenograft tumors initiated with Aldefluor(+) cells (Fig. 4D). Thus, prodigiosin is a potent p53 pathway restoring agent in CRCSCs.

Prodigiosin-mediated anti-CRCSC effect involves p53 pathway restoration via p73

Prodigiosin induced cell death in colonospheres (Fig. 1D) and reduced the viability of CRCSCs (Fig. 2D). Western blot analysis revealed that prodigiosin induced the markers of cell death cleaved PARP and cleaved caspase-8 in CRCSCs (Fig. 5A). Time-course analysis of prodigiosin-mediated effects on cell

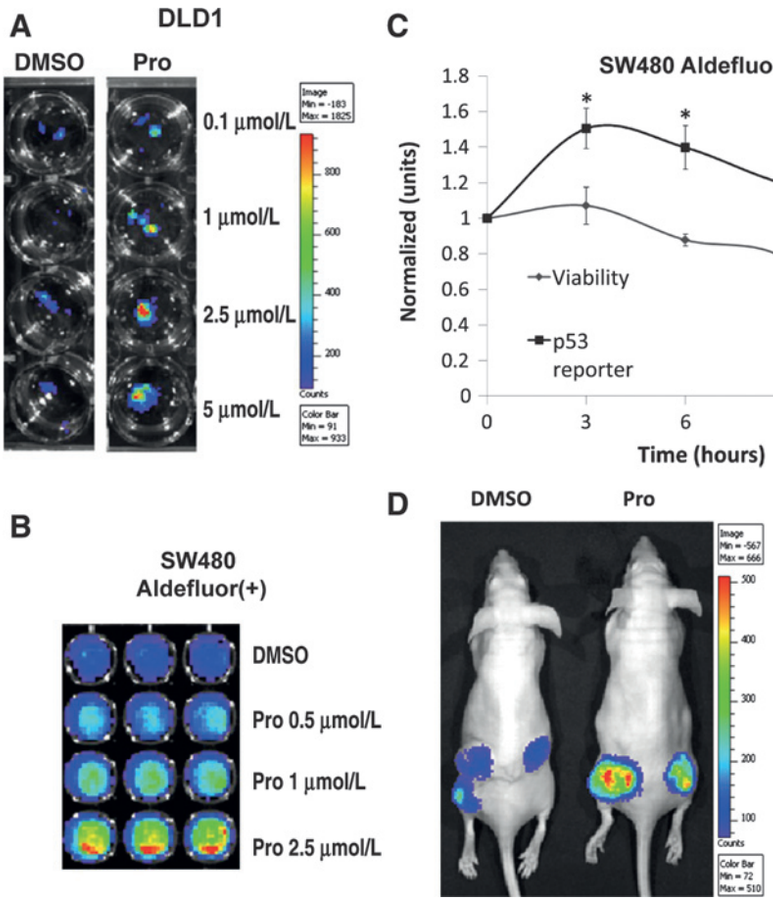

Figure 4.
Prodigiosin restores the p53 pathway in CRCSCs *in vitro* and *in vivo*. A, DLD1 cells expressing the p53-responsive luciferase reporter were plated for colonosphere formation. Colonospheres were treated with DMSO or indicated doses of prodigiosin (Pro) for 4 hours and luciferase reporter activity was measured. B, SW480 Aldefluor(+) cells expressing the p53-responsive luciferase reporter were plated, treated with DMSO or indicated doses of prodigiosin for 8 hours, and luciferase reporter activity was measured. C, SW480 Aldefluor(+) cells expressing the p53-responsive luciferase reporter were plated and treated with DMSO or 1 μmol/L prodigiosin. Luciferase reporter activity and cell viability was measured at the indicated time points. D, SW480 Aldefluor(+) cells were injected subcutaneously into the flanks of athymic nude mice. Upon tumor formation, mice were administered DMSO or prodigiosin 5 mg/kg (i.p.), and luciferase reporter activity was measured after 12 hours.

viability, cell death, and p53 pathway restoration indicated that prodigiosin-mediated p53 pathway restoration was followed by a reduction in cell viability and an increase in cell death (Figs. 4C and 5A). Prodigiosin-mediated inhibition of holoclone (Supplementary Fig. S1C) and colonosphere formation (Fig. 1A) was observed in p53 wild-type (HCT116, RKO) and p53 mutant (DLD1, SW480, SW620) cells. Also, p53 loss did not abrogate prodigiosin-mediated inhibition of colonosphere formation in HCT116 cells (Supplementary Fig. S4B). These results indicate that prodigiosin-mediated CRCSC cell death and inhibition of self-renewal involves p53 pathway restoration and is independent of colorectal cancer p53 status.

We have previously shown that prodigiosin-mediated p53 pathway restoration in colorectal cancer cells involves p73 activation (35). To confirm the mechanism of prodigiosin-mediated p53 pathway transcriptional activation in CRCSCs, we examined the protein levels of p73 and its target genes. Prodigiosin upregulated the levels of p73 and p53/p73 common target gene p21 in Aldefluor(+) cells (Fig 5A). We also observed that prodigiosin elevated the levels of Jagged-1 (Fig. 5A), a p53-independent p73 target gene (6). Prodigiosin-mediated p21 induction was reduced upon p73 knockdown (Fig. 5B). Clearly, prodigiosin-mediated p53 pathway restoration is p73 dependent. To further confirm that the prodigiosin-mediated anti-CRCSC effect is dependent on p73, we used p73 knockdown colorectal cancer cells. Knockdown of p73 significantly reduced prodigiosin-mediated colonosphere inhibition in DLD1 and SW480 cells (Fig. 5C and D and Supplementary Fig. S4C). Thus, prodigiosin-mediated cell death and inhibition of self-renewal involve p53 pathway restoration via p73 activation.

Prodigiosin-mediated p53 pathway restoration, cell death, and anti-CRCSC effect involves inhibition of ΔNp73

Previously, we have reported that prodigiosin further potentiates the effects of p73 activation via disruption of mutant p53 mediated inhibition of p73 (35). Prodigiosin also reduced the levels of the oncogenic isoform ΔNp73 (Fig. 6A) known to inhibit the effects of p73 and p53 (12, 13). Interestingly, SW480 cells expressed lower endogenous ΔNp73 levels as compared with DLD1 cells (Supplementary Fig. S4D). Accordingly, IC_{50} for prodigiosin in SW480 cells was lower compared with DLD1 cells (35). Prodigiosin-mediated inhibition of SW480 colonospheres was greater than that observed with DLD1 colonospheres (Fig. 1A). In addition, SW480 Aldefluor(+) xenograft tumors were more sensitive to antitumor effects of prodigiosin compared with DLD1 Aldefluor(+) tumors (Fig. 3A and Supplementary Fig. S2B). Together, the data indicates that ΔNp73 levels can help predict sensitivity to prodigiosin. To conclusively demonstrate the importance of ΔNp73 downregulation to the overall anticancer efficacy of prodigiosin, we explored the effect of ΔNp73 overexpression on prodigiosin-mediated p53 pathway restoration, cell death, and anti-CRCSC effects. ΔNp73 overexpression significantly reduced prodigiosin-mediated cell death in HCT116 and DLD1 cells (Fig. 6B and Supplementary Fig. S5B). Prodigiosin-mediated

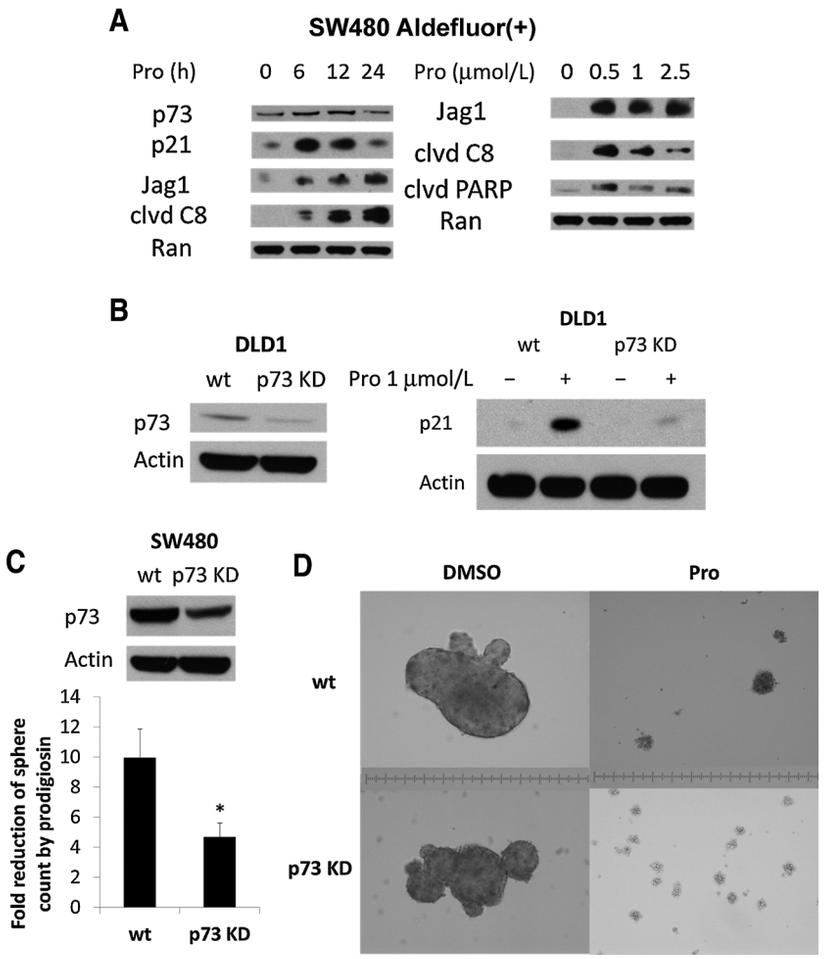

Figure 5.
Prodigiosin-mediated anti-CRCSC effect involves p53 pathway restoration via p73. A, SW480 Aldefluor(+) cells were treated with DMSO or 1 μmol/L prodigiosin (Pro) for indicated time points or with indicated doses (μmol/L) of prodigiosin for 24 hours. Western blot analysis was performed for p73, p21, Jagged-1 (Jag1), cleaved (clvd) caspase-8 (C8), cleaved PARP, and Ran. B, DLD1 wild-type (wt) and stable p73 knockdown (KD) cells were treated with DMSO or 1 μmol/L prodigiosin for 12 hours. Western blot analysis was performed for p73, p21, and actin. C, SW480 wt and p73 knockdown cells (Western blot analysis validation shown) were plated for colonosphere formation in the presence of DMSO or 0.5 μmol/L prodigiosin and counted after 4 days. *, $P < 0.05$. D, DLD1 wt and p73 knockdown cells were plated for colonosphere formation in the presence of DMSO or 1 μmol/L prodigiosin and imaged (magnification, ×10) after 3 days. Each division on the scale is 10 μm.

p53 reporter activation was also reduced upon ΔNp73 overexpression (Supplementary Fig. S5A). Interestingly, ΔNp73 overexpression increased the sphere formation of DLD1 cells (Fig. 6E and Supplementary Fig. S5C). ΔNp73 overexpression decreased prodigiosin-mediated inhibition of sphere formation. Thus, ΔNp73 inhibition is important for prodigiosin-mediated p53 pathway restoration, cell death, and anti-CRCSC effects.

Prodigiosin-mediated p53 pathway restoration via p73 activation and ΔNp73 inhibition involves upstream c-Jun activation

c-Jun has been shown to regulate both p73 and ΔNp73 levels under conditions of cellular stress (17, 18). We investigated whether prodigiosin-mediated p53 pathway restoration via p73 and ΔNp73 involves upstream effects on c-Jun. Prodigiosin upregulated c-Jun and induced phosphorylation of c-Jun in sorted Aldefluor(+) CRCSCs (Fig. 6C). Prodigiosin elevated c-Jun levels in both the cytoplasm and nucleus, which also correlated with p21 upregulation and nuclear translocation (Supplementary Fig. S6A). To determine the significance of c-Jun upregulation in prodigiosin-mediated antitumor effects, we pursued knockdown of c-Jun levels. c-Jun knockdown reduced prodigiosin-mediated p73 upregulation and ΔNp73 downregulation (Fig. 6D). Thus, c-Jun upregulation is an important upstream event for prodigiosin-mediated effects on p73 and ΔNp73 (Fig. 6F). To further validate the mechanism, we determined the effects of c-Jun knockdown on p53 pathway restoration and cell death by prodigiosin. c-Jun knockdown reduced both prodigiosin-mediated p53 reporter activation and cell death (Supplementary Fig. S6B and C). Clearly, p53 pathway restoration and antitumor effects of prodigiosin via p73 occur in a c-Jun–dependent manner (Fig. 6F).

Discussion

Self-renewing CRCSCs are a subpopulation within colorectal cancer responsible for long-term tumor maintenance (19, 20), therapy resistance (21), relapse (23), and metastasis (22, 24). CRCSCs could arise from normal stem cells (45, 46) or by dedifferentiation of non-CRCSC tumor cells (25, 26). For successful long-term colorectal cancer therapy, it is critical to target both CRCSC and non-CRCSC tumor cells. In our study, we have modeled the self-renewal and chemotherapy resistance of CRCSCs *in vitro* and *in vivo* using colonosphere culture, marker-based flow cytometry sorting of CRCSCs, CRCSC-mediated xenograft tumor growth and passage. In addition, we have characterized a potent small molecule that targets both CRCSCs and bulk tumor cells. Small-molecule prodigiosin decreased CRCSC

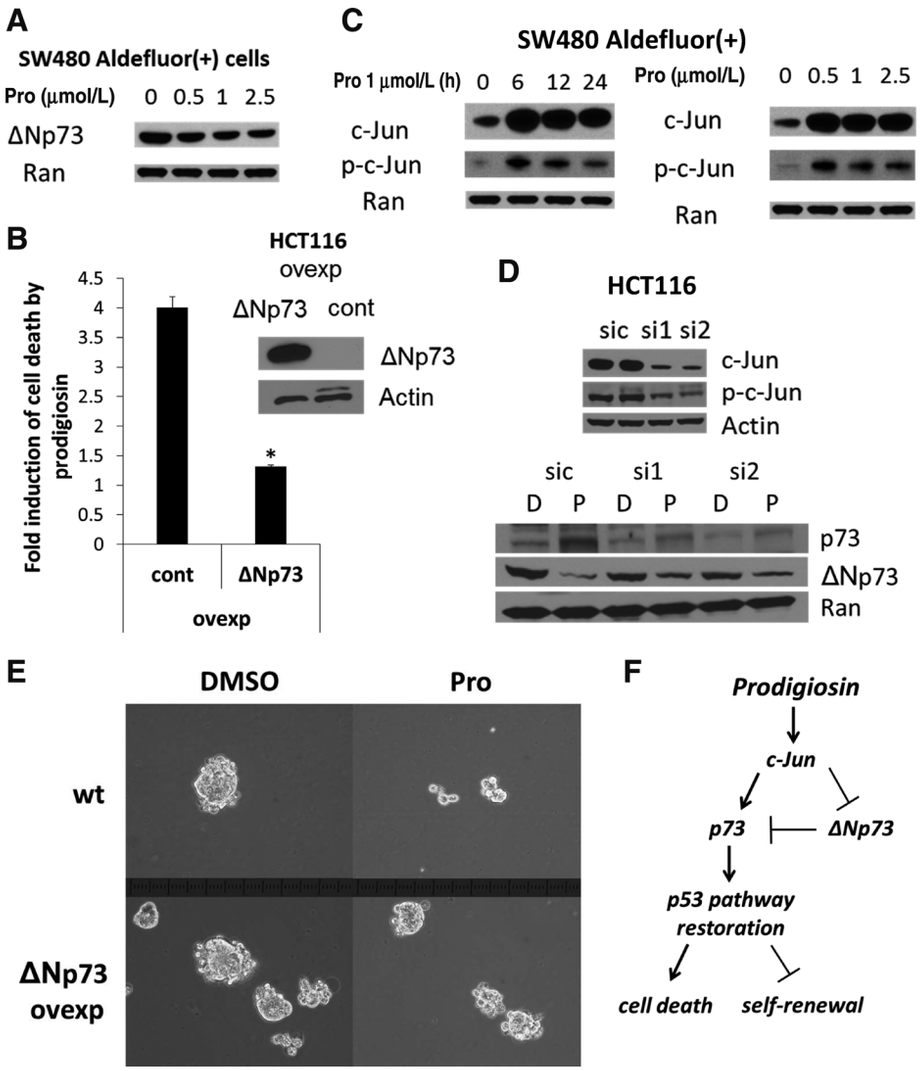

Figure 6.
Prodigiosin-mediated p53 pathway restoration, cell death, and anti-CRCSC effects involve c-Jun activation and inhibition of ΔNp73. A, SW480 Aldefluor(+) cells were treated with DMSO or indicated doses of prodigiosin (Pro) for 24 hours. Western blot analysis was performed for ΔNp73 and Ran. B, HCT116 cells were transfected with control (cont) or ΔNp73 overexpression (ovexp) plasmids (Western blot analysis validation shown) and treated with DMSO or 1 μmol/L prodigiosin for 48 hours. Cell death was determined by sub-G_1 analysis. *, $P < 0.003$. C, SW480 Aldefluor(+) cells were treated with DMSO or 1 μmol/L prodigiosin (Pro) for indicated time points or with indicated doses of prodigiosin (μmol/L) for 24 hours. Western blot analysis was performed for c-Jun, phospho (p)-c-Jun and Ran. D, HCT116 cells were transfected with siRNA control (sic) or c-Jun siRNA (si1 and si2; Western blot validation shown) and treated with DMSO (D) or 1 μmol/L prodigiosin (P) for 12 hours. Western blot analysis was performed for p73, ΔNp73, and Ran. E, DLD1 cells were transfected with control (wt) or ΔNp73 overexpression (ovexp) plasmids, plated for colonosphere formation in the presence of DMSO or 0.5 μmol/L prodigiosin and imaged (magnification, ×20) after 2 days. Each division on the scale is 10 μm. F, prodigiosin-mediated cell death and inhibition of self-renewal involve p53 pathway restoration via c-Jun-mediated p73 activation and inhibition of ΔNp73.

markers and reduced colonosphere formation of colorectal cancer cells as well as 5-FU–resistant sorted CRCSCs. Prodigiosin reduced the viability of both CRCSCs and non-CRCSCs whereas 5-FU targeted only non-CRCSCs. Prodigiosin also prevented CRCSC-initiated xenograft tumor growth and passage. It is important to note that prodigiosin-mediated anti-CRCSC effects involve inhibition of self-renewal as well as cell death induction. We observed that removal of prodigiosin after a short exposure could still inhibit colonosphere formation. Also, tumor growth did not resume even in the absence of continued prodigiosin treatment. These findings suggest that prodigiosin has a long-term effect on CRCSC self-renewal. Previous studies have shown that prodigiosin is retained in the mitochondria in cancer cells (47) and is not a substrate for multidrug resistance efflux pumps (48). These properties of prodigiosin could contribute to the sustained effects observed. We have previously demonstrated that prodigiosin does not induce DNA damage at the therapeutic doses tested, is more toxic to cancer cells compared with normal cells and does not alter the body weight of mice (35). In the current study, we further confirmed our previous results and showed that prodigiosin targets CRCSCs without toxic effects using serum chemistry analysis and tissue histology. Our data is consistent with other reports of lack of toxicity to normal cells with prodigiosin (49, 50). Thus, prodigiosin targets CRCSC self-renewal *in vitro* and *in vivo* without toxic effects.

Loss of p53 function elevates CRCSCs and p53 pathway restoration targets CRCSC self-renewal (27); however, the effects of p73 restoration on CRCSCs remains unknown. We have previously described prodigiosin as a potent p53 pathway restoring agent that activates p73 (35). In the current study, we have demonstrated that the anti-CRCSC effects of prodigiosin involve p53 pathway restoration in a p73-dependent manner. Prodigiosin targeted CRCSC self-renewal independent of p53 status and restored p53 pathway transcription in chemotherapy-resistant CRCSCs. Knockdown of p73 reduced prodigiosin-mediated p53 pathway restoration and anti-CRCSC effects. Thus, small-molecule–mediated p73 restoration can target both chemotherapy-resistant CRCSCs and non-CRCSCs in tumors with wild-type as well as mutant p53. Our results are in agreement with the study by Hu and colleagues demonstrating that p73 overexpression

increases cisplatin-mediated apoptosis in glioblastoma stem cells (34). No prior study has demonstrated the use of prodigiosin or small-molecule–mediated p73 activation for targeting cancer stem cells.

Previously, we have reported that prodigiosin further potentiates the effects of p73 activation via disruption of mutant p53-mediated inhibition of p73 (35). In this study, we identified an additional mechanism by which prodigiosin potentiates the effects of p73 activation. ΔNp73 is known to inhibit the effects of p73 and p53 (12, 13). Prodigiosin increased the levels of p73 and reduced the levels of oncogenic ΔNp73 in CRCSCs. Using ΔNp73 overexpression, we further demonstrated that inhibition of ΔNp73 is an integral part of prodigiosin-mediated p53 pathway restoration, cell death, and inhibition of self-renewal. Thus, ΔNp73 could potentially serve as a useful biomarker of response for prodigiosin and monitoring ΔNp73 levels may help with patient stratification. Interestingly, our data indicates that overexpression of ΔNp73 promotes self-renewal of colorectal cancer cells. Further work is needed to understand the role of p73 and ΔNp73 in regulation of CRCSC self-renewal.

AP-1 transcription factor family member c-Jun has been shown to regulate levels of p73 and ΔNp73 under conditions of cellular stress (17, 18). In this study, we have demonstrated c-Jun as an important upstream nodal mechanism of prodigiosin-mediated p53 pathway restoration via p73. Prodigiosin elevated levels of c-Jun in the nucleus, indicating that c-Jun transcriptionally regulates p73 and ΔNp73 levels. Transcriptional regulation of p73 and ΔNp73 by c-Jun could potentially occur via effects on Yes-associated protein (51) or the proteasome (18).

Although prodigiosin mediated effects on CRCSCs are dependent on p73, other mechanisms independent of p53 pathway restoration such as inhibition of Akt likely contribute to the anti-CRCSC effects (52). Further studies will be required to assess the p53 pathway independent anti-CRCSC mechanisms of prodigiosin.

Disclosure of Potential Conflicts of Interest

W.S. El-Deiry has ownership interest (including patents) in p53 therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: V.V. Prabhu, B. Hong, S. Zhang, W.S. El-Deiry
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V.V. Prabhu, J.E. Allen, W.S. El-Deiry
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V.V. Prabhu, J.E. Allen, W.S. El-Deiry
Writing, review, and/or revision of the manuscript: V.V. Prabhu, J.E. Allen, S. Zhang, D.T. Dicker, W.S. El-Deiry
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.V. Prabhu, S. Zhang, A.R. Lulla, D.T. Dicker, W.S. El-Deiry
Study supervision: W.S. El-Deiry

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References

1. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. CA Cancer J Clin 2014;64:104–17.
2. Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Willson JK, et al. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Res 1990;50:7717–22.
3. Hong B, van den Heuvel AP, Prabhu VV, Zhang S, El-Deiry WS. Targeting tumor suppressor p53 for cancer therapy: strategies, challenges and opportunities. Curr Drug Targets 2014;15:80–9.
4. Bunz F, Hwang PM, Torrance C, Waldman T, Zhang Y, Dillehay L, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 1999;104:263–9.
5. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, et al. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997;90:809–19.
6. Fontemaggi G, Kela I, Amariglio N, Rechavi G, Krishnamurthy J, Strano S, et al. Identification of direct p73 target genes combining DNA microarray and chromatin immunoprecipitation analyses. J Biol Chem 2002;277:43359–68.
7. Sunahara M, Ichimiya S, Nimura Y, Takada N, Sakiyama S, Sato Y, et al. Mutational analysis of the p73 gene localized at chromosome 1p36.3 in colorectal carcinomas. Int J Oncol 1998;13:319–23.
8. Wang W, Kim SH, El-Deiry WS. Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. Proc Natl Acad Sci U S A 2006;103:11003–8.
9. Lu C, Wang W, El-Deiry WS. Non-genotoxic anti-neoplastic effects of ellipticine derivative NSC176327 in p53-deficient human colon carcinoma cells involve stimulation of p73. Cancer Biol Ther 2008;7:2039–46.
10. Kravchenko JE, Ilyinskaya GV, Komarov PG, Agapova LS, Kochetkov DV, Strom E, et al. Small-molecule RETRA suppresses mutant p53-bearing cancer cells through a p73-dependent salvage pathway. Proc Natl Acad Sci U S A 2008;105:6302–7.
11. Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC, et al. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. Genes Dev 2008;22:2677–91.
12. Stiewe T, Theseling CC, Putzer BM. Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis. J Biol Chem 2002;277:14177–85.
13. Wilhelm MT, Rufini A, Wetzel MK, Tsuchihara K, Inoue S, Tomasini R, et al. Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway. Genes Dev 2010;24:549–60.
14. Soldevilla B, Diaz R, Silva J, Campos-Martin Y, Munoz C, Garcia V, et al. Prognostic impact of DeltaTAp73 isoform levels and their target genes in colon cancer patients. Clin Cancer Res 2011;17:6029–39.
15. Di Como CJ, Gaiddon C, Prives C. p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Mol Cell Biol 1999;19:1438–49.
16. Bergamaschi D, Gasco M, Hiller L, Sullivan A, Syed N, Trigiante G, et al. p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell 2003;3:387–402.
17. Toh WH, Siddique MM, Boominathan L, Lin KW, Sabapathy K. c-Jun regulates the stability and activity of the p53 homologue, p73. J Biol Chem 2004;279:44713–22.
18. Dulloo I, Gopalan G, Melino G, Sabapathy K. The antiapoptotic DeltaNp73 is degraded in a c-Jun-dependent manner upon genotoxic stress through the antizyme-mediated pathway. Proc Natl Acad Sci U S A 2010;107:4902–7.

19. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–10.
20. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111–5.
21. Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 2007;1:389–402.
22. Pang R, Law WL, Chu AC, Poon JT, Lam CS, Chow AK, et al. A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell 2010;6:603–15.
23. Merlos-Suarez A, Barriga FM, Jung P, Iglesias M, Cespedes MV, Rossell D, et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell 2011;8:511–24.
24. Todaro M, Gaggianesi M, Catalano V, Benfante A, Iovino F, Biffoni M, et al. CD44v6 is a marker of constitutive and reprogrammed cancer stem cells driving colon cancer metastasis. Cell Stem Cell 2014;14:342–56.
25. Schwitalla S, Fingerle AA, Cammareri P, Nebelsiek T, Goktuna SI, Ziegler PK, et al. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell 2013;152:25–38.
26. Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 2010;12:468–76.
27. Prabhu VV, Allen JE, Hong B, Zhang S, Cheng H, El-Deiry WS. Therapeutic targeting of the p53 pathway in cancer stem cells. Expert Opin Ther Targets 2012;16:1161–74.
28. Li L, Wang L, Wang Z, Ho Y, McDonald T, Holyoake TL, et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell 2012;21:266–81.
29. Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell 2009;138:1083–95.
30. Talos F, Abraham A, Vaseva AV, Holembowski L, Tsirka SE, Scheel A, et al. p73 is an essential regulator of neural stem cell maintenance in embryonal and adult CNS neurogenesis. Cell Death Differ 2010;17:1816–29.
31. Agostini M, Tucci P, Chen H, Knight RA, Bano D, Nicotera P, et al. p73 regulates maintenance of neural stem cell. Biochem Biophys Res Commun 2010;403:13–7.
32. Gonzalez-Cano L, Herreros-Villanueva M, Fernandez-Alonso R, Ayuso-Sacido A, Meyer G, Garcia-Verdugo JM, et al. p73 deficiency results in impaired self renewal and premature neuronal differentiation of mouse neural progenitors independently of p53. Cell Death Dis 2010;1:e109.
33. Lin Y, Cheng Z, Yang Z, Zheng J, Lin T. DNp73 improves generation efficiency of human induced pluripotent stem cells. BMC Cell Biol 2012;13:9.
34. Hu X, Wu N, Xia P, Yu S, Sun F, Chen J. Correlation between low-level expression of the tumor suppressor gene TAp73 and the chemoresistance of human glioma stem cells. Cancer Chemother Pharmacol 2012;69:1205–12.
35. Hong B, Prabhu VV, Zhang S, van den Heuvel AP, Dicker DT, Kopelovich L, et al. Prodigiosin rescues deficient p53 signaling and antitumor effects via upregulating p73 and disrupting its interaction with mutant p53. Cancer Res 2014;74:1153–65.
36. Zhang S, Zhou L, Hong B, van den Heuvel AP, Prabhu VV, Warfel NA, et al. Small-molecule NSC59984 restores p53 pathway signaling and antitumor effects against colorectal cancer via p73 activation and degradation of mutant p53. Cancer Res 2015;75:3842–52.
37. Prabhu VV, Allen JE, Dicker DT, El-Deiry WS. Small-molecule ONC201/TIC10 targets chemotherapy-resistant colorectal cancer stem-like cells in an Akt/Foxo3a/TRAIL-dependent manner. Cancer Res 2015;75:1423–32.
38. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, et al. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med 2011;17:211–5.
39. Kanwar SS, Yu Y, Nautiyal J, Patel BB, Majumdar AP. The Wnt/beta-catenin pathway regulates growth and maintenance of colonospheres. Mol Cancer 2010;9:212.
40. Healey E, Stillfried GE, Eckermann S, Dawber JP, Clingan PR, Ranson M. Comparative effectiveness of 5-fluorouracil with and without oxaliplatin in the treatment of colorectal cancer in clinical practice. Anticancer Res 2013;33:1053–60.
41. Huang EH, Hynes MJ, Zhang T, Ginestier C, Dontu G, Appelman H, et al. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res 2009;69:3382–9.
42. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 2007;104:10158–63.
43. O'Brien CA, Kreso A, Ryan P, Hermans KG, Gibson L, Wang Y, et al. ID1 and ID3 regulate the self-renewal capacity of human colon cancer-initiating cells through p21. Cancer Cell 2012;21:777–92.
44. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 2004;117:927–39.
45. Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, et al. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 2009;457:603–7.
46. Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science 2012;337:730–5.
47. Francisco R, Perez-Tomas R, Gimenez-Bonafe P, Soto-Cerrato V, Gimenez-Xavier P, Ambrosio S. Mechanisms of prodigiosin cytotoxicity in human neuroblastoma cell lines. Eur J Pharmacol 2007;572:111–9.
48. Elahian F, Moghimi B, Dinmohammadi F, Ghamghami M, Hamidi M, Mirzaei SA. The anticancer agent prodigiosin is not a multidrug resistance protein substrate. DNA Cell Biol 2013;32:90–7.
49. Montaner B, Navarro S, Pique M, Vilaseca M, Martinell M, Giralt E, et al. Prodigiosin from the supernatant of Serratia marcescens induces apoptosis in haematopoietic cancer cell lines. Br J Pharmacol 2000;131:585–93.
50. Yamamoto C, Takemoto H, Kuno K, Yamamoto D, Tsubura A, Kamata K, et al. Cycloprodigiosin hydrochloride, a new H(+)/Cl(-) symporter, induces apoptosis in human and rat hepatocellular cancer cell lines in vitro and inhibits the growth of hepatocellular carcinoma xenografts in nude mice. Hepatology 1999;30:894–902.
51. Danovi SA, Rossi M, Gudmundsdottir K, Yuan M, Melino G, Basu S. Yes-associated protein (YAP) is a critical mediator of c-Jun-dependent apoptosis. Cell Death Differ 2008;15:217–9.
52. Chang CC, Chen WC, Ho TF, Wu HS, Wei YH. Development of natural anti-tumor drugs by microorganisms. J Biosci Bioeng. 2011;111:501–11.