Synergism between vitamin D and secreted protein acidic and rich in cysteine–induced apoptosis and growth inhibition results in increased susceptibility of therapy-resistant colorectal cancer cells to chemotherapy

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Abstract

Patients with advanced colorectal cancer continue to have poor outcomes because of therapy-refractory disease. We previously showed that secreted protein acidic and rich in cysteine (SPARC) gene and protein could function as a chemotherapy sensitizer by enhancing tumor regression in response to radiation and chemotherapy in tumor xenograft models of chemotherapy-resistant tumors. This function of SPARC was gleamed from a microarray analysis that also revealed down-regulation of the vitamin D receptor (*VDR*) in therapy-refractory colorectal cancer cells. This study examines the potential synergistic effect of SPARC and vitamin D, which up-regulates VDR, in enhancing chemotherapy response in colorectal cancer. Using MIP101 colorectal cancer cell lines and *SPARC*-overexpressing MIP101 cells, we were able to show that, in the presence of SPARC, exposure to low doses of 1α,25-dihydroxyvitamin D_3 significantly reduces cell viability, enhances chemotherapy-induced apoptosis, and inhibits the growth of colorectal cancer cells. Moreover, in tumor xenograft mouse models, up-regulation of VDR was seen in tumors that had the greatest regression following treatment that combined SPARC with chemotherapy. Therefore, our findings reveal a synergistic effect between SPARC and low doses of 1α,25-dihydroxyvitamin D_3 that further augments the sensitivity of tumors to chemotherapy. This combination may prove to be a useful adjunct in the treatment of colorectal cancer, especially in those patients with therapy-refractory disease. [Mol Cancer Ther 2007;6(1):309–17]

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Introduction

Colorectal cancer is one of the leading causes of cancer-related deaths worldwide (1). For the last 40 years, the only treatment available consisted primarily of 5-fluorouracil (5-FU)–based therapy. The recent introduction of newer agents for the treatment of stage IV colorectal cancer, such as irinotecan (topoisomerase-1 inhibitor) and oxaliplatin (DNA-damaging agent), has now expanded our therapeutic options. Improved response rates of 40% to 50% have been observed with these drugs in combination with 5-FU in comparison with 10% to 15% response rates for 5-FU/leucovorin alone (2, 3). Unfortunately, most patients who initially respond to therapy later relapse due to therapy-refractory disease, which accounts for our inability to improve the survival of patients diagnosed with advanced colorectal cancer despite the availability of newer therapies (2, 3). It is clear that therapies that can overcome chemotherapy resistance will have a greater impact in improving the survival of patients diagnosed with advanced cancers.

Over the years, considerable evidence has accumulated to suggest a beneficial effect of vitamin D in preventing and treating colorectal cancers (4–9). The initial association between vitamin D and colon cancer was provided by epidemiologic studies showing an inverse correlation between the incidence of colon cancer and exposure to sunlight, vitamin D intake, and serum levels of vitamin D metabolites (9, 10). 1α,25-Dihydroxyvitamin D_3 (1,25-D3), an active metabolite of vitamin D and a member of the secosteroid hormone family, is classically known for its physiologic role in calcium homeostasis and bone mineralization. It mediates its action by interacting with the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. Separate from this classic role, it is negatively involved in cell proliferation of normal and malignant cells *in vitro* (11–15). Its therapeutic potential in cancer has been assessed in a large number of studies, but unfortunately, its major limitation for its clinical use is the development of hypercalcemia, a major and serious side effect. As a consequence, newer analogues with less calcemic effects and similar antiproliferative properties have been developed and tested in recent years (16–23).

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Secreted protein acidic and rich in cysteine (SPARC; osteonectin, BM-40) belongs to a family of matricellular proteins that include thrombospondins, tenascin, and osteopontin. Initially found to be important in wound healing, considerable evidence has now accumulated that indicates a role of SPARC in tumorigenesis. Although variable expression has been observed in different types of cancers, this 42-kDa glycoprotein may function as a tumor suppressor. Recent studies have shown tumor growth–retarding properties in pancreatic, ovarian, and breast cancers, either following exogenous exposure or by overexpressing this protein *in vitro* (24–26). In animal models, SPARC is capable of inhibiting the growth of neuroblastomas (27) while enhancing tumor growth in an environment devoid of SPARC, as seen in tumor xenograft models in SPARC-null mice (28, 29). More recently, our laboratory showed chemosensitizing properties of SPARC, wherein the administration of the exogenous protein in combination with 5-FU promotes greater tumor regression in xenograft mouse models (30). SPARC was identified as a potential chemosensitizer from a genome-wide microarray analysis that provided a signature profile of chemotherapy resistance. This same analysis also revealed a concomitant decrease in VDR expression in chemotherapy-resistant MIP101 colorectal cancer cells. We therefore wondered whether up-regulation of VDR following vitamin D exposure could enhance the effect of SPARC in improving sensitivity to chemotherapy in resistant colorectal cancer cells. This study assesses the potential synergism between SPARC and low-dose vitamin D in enhancing chemotherapy sensitivity in colorectal cancer cells.

Materials and Methods

Cells

Chemotherapy-sensitive human colorectal cancer cell lines MIP101 (31) and HCT 116 and cells resistant to 5-FU (MIP/5-FU, HCT/5-FU), CPT-11 (MIP/CPT, HCT/CPT), and cisplatin (MIP/cisplatin, HCT/cisplatin) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Invitrogen Life Technologies, Inc., Burlington, Ontario, Canada). HCT 116 resistant cell lines were developed following long-term incremental exposure to individual chemotherapies and have an IC_{50} ~6-fold higher than the sensitive cells. MIP101 cells stably transduced with *SPARC* (MIP/SP) and its empty vector control (MIP/Zeo) were similarly maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1% Zeocin. All cells were incubated in a humidified incubator at 37°C and 5% CO_2.

Reverse Transcription-PCR

Total RNA was extracted from cultured cells, at 75% confluence and under similar culture conditions, using Trizol reagent (Invitrogen). Reverse transcription-PCR was done using Avian RT (Sigma, Oakville, Ontario, Cancada). The following primers were used in this study: SPARC, 5′-CATCTTCCCTGTACACTG-3′ (sense) and 5′-ATGGGGATGAGGGGGAG-3′ (antisense); GAPDH, 5′-CTCTCTGCTCCTCCTGTTCGACAG-3′ (sense) and 5′-AGGGGTCTTACTCCTTGGAGGCCA-3′ (antisense); VDR, 5′-AGGCTGCAAAGGCTTCTTCA-3′ (sense) and 5′-AGGGTCATCTGAATCTTCTT-3′ (antisense); and β-actin, 5-GCCACGGCTGCTTCCAG-3′ (sense) and 5′-GGCGTACAGGTCTTTGC-3′ (antisense). Reaction was set at 50°C × 1 h; followed by 32 to 42 cycles of 94°C × 1 min, 65°C × 1 min, 72°C × 2 min; followed by 72°C × 10 min. PCR products were separated on 1% agarose gel electrophoresis.

Cell Viability and Colony-Forming Assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) assay (Promega, Madison, WI). MIP101, MIP/5-FU, MIP/CPT, MIP/SP, and MIP/Zeo cells incubated at a density of 4,500 per well (96-well plate) were exposed to the following concentrations of cholecalciferol (1,25-D3, dissolved in ethanol; Sigma) for 24 h: 1.25, 2.4, 6.2, 37.5, or 62.5 nmol/L; or ergocalciferol (dissolved in ethanol; Sigma) at 1 or 2.5 μmol/L (cells in the control group were exposed to the ethanol vehicle only); followed by another 24-h exposure to either 500 or 1,000 μmol/L of 5-FU, or 200 μmol/L CPT-11. After addition of MTS, cells were incubated for an additional 2 h at 37 °C with 5% CO_2, and the absorbance of each well at 490 nm was measured in a 96-well plate reader (Versa Max, version 4.8, Molecular Devices Co., Sunnyvale, CA) according to the manufacturer's instructions. For colony-forming assay, cells were seeded at 1,000 per well in a 48-well plate. Forty-eight hours later, cells were incubated with incremental concentrations of 5-FU (0, 10, 100, or 1,000 μmol/L) or CPT-11 (0, 1, 10, or 100 μmol/L) for 4 days, washed with DMEM, and incubated in fresh medium containing the appropriate concentrations of chemotherapy for an additional 7 days, followed by staining with 0.2% crystal violet. The number of colonies formed in the treated group was calculated based on the colonies formed from the control, untreated cells. There were three replicates for each experiment.

Detection of Apoptosis by Terminal Deoxyribonucleotidyl Transferase–Mediated dUTP Nick End Labeling (TUNEL) Assay

Cells were seeded (80,000 per well) in a 48-well plate for 48 h in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin; washed and incubated in serum-free conditioned medium supplemented with 4 mmol/L glutamine (VP-SFM, Invitrogen); followed by incubation with 2.4 nmol/L 1,25-D3 for a 24-h period before treatment with 1,000 μmol/L 5-FU for an additional 12 h. Cells were collected (all free-floating or adherent cells after trypsinization) and attached to microscopy slides by Shandon Cytospin (Thermo Fisher Scientific, Ottawa, Ontario, Canada) at 3,000 rpm for 10 min. Samples were briefly air-dried (3 min) before fixation with 4% paraformaldehyde and processed for labeling with Apoptosis Detection Kit (Promega) based on the manufacturer's protocol. The number of TUNEL–positive cells in each treatment group was determined based on cell counts from four different fields containing at least 100 cells per field at ×40 magnification (n = 3 separate experiments).

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Cell Cycle Analysis
MIP/Zeo and MIP/SP cells seeded at 200,000 per well in a six-well plate in DMEM (FBS 5%) were subjected to cell cycle synchronization with double thymidine block [thymidine 2 mmol/L (Sigma) in DMEM supplemented with FBS 2%] as previously described (30). Following an initial 16-h thymidine block, cells were released in DMEM (FBS 10%) for 12 h, which was then followed by a second 14-h thymidine block. Cells released from this block were collected at timed intervals and processed for cell cycle analysis: cells were fixed in 80% ethanol for 30 min on ice, followed by incubation with propidium iodide 500 μg/mL (Sigma) and RNase 50 μg/mL (Sigma) in PBS for 30 min at 37°C, and analyzed with Becton Dickinson (Mississauga, Ontario, Canada) FACSCalibur (n = 3 independent experiments).

Western Blot Analysis
Cells were collected by centrifugation and total protein was extracted with CHAPS cell lysis buffer [0.1% CHAPS, 20 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL aprotinin, 50 mmol/L PIPES/HCl (pH 6.5), 5 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride]. Lysates were quickly frozen-thawed thrice and centrifuged at 10,000 × g for 10 min. Soluble proteins were quantitated by the Bradford method (Pierce, Rockford, IL). Total protein (30–45 μg) was resolved by 10% or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were reversibly stained with 0.1% Ponceau red solution, then blocked with 5% Blotto [5% nonfat dry milk dissolved in TBS (25 mmol/L Tris) solution containing 0.1% Tween] as previously described (30). Membranes were incubated overnight at 4°C with the primary antibodies in 5% Blotto (all primary antibodies were diluted 1:1,000), washed, then incubated with an appropriate secondary antibody (either antimouse or antirabbit conjugated to horseradish peroxidase; dilution 1:20,000) for 2 h at room temperature. The membranes were again washed in Blotto thrice before being developed by an enhanced chemiluminescence detection system according to the manufacturer's recommendations (West Dura reagent, Pierce). The following antibodies were used: SPARC (1 μg/mL; Haematologic Technologies, Inc., Essex Junction, VT); α-tubulin (0.2 μg/mL; Sigma); VDR (1 μg/mL; Affinity Bioreagents, Golden, CO); phosphatase and tensin homologue, p-Akt (serine/threonine), Bad, phosphorylated cyclin-dependent kinase 2 (p-cdk-2), cyclin-D1, and p-Rb (1:1,000 dilution; Cell Signaling Technologies, Danvers, MA).

Immunofluorescence Staining
Cells seeded on coverslips in a 24-well plate were incubated with 2.4 nmol/L 1,25-D3 for 24 h followed by treatment with 500 μmol/L 5-FU for an additional 24 h, fixed in 4% paraformaldehyde for 20 min and methanol (−20°C) for 5 min, and washed in PBS twice (3 min), then in PBS containing 0.1% Triton X-100 (Sigma) for an additional 5 min. Cells were blocked with 2% bovine serum and incubated with primary antibodies overnight at 4°C, rinsed in PBS twice (3 min), followed by incubation with secondary antibodies at 37°C for 20 min, rinsed in PBS, and stained with 4′,6-diamidino-2-phenylindole. Primary antibodies used included mouse α-SPARC (4 μg/mL) and mouse α-VDR (5 μg/mL). For immunohistochemistry, paraffin-embedded tumors harvested from xenografts from animals (NIH nude mice, 6 weeks old; Taconic Laboratories, Hudson, NY) were used. MIP101 cells (2×10^6) were injected into the left flank as previously described (30). Once tumors reached 100 cm^3, animals were treated with chemotherapy using a 3-week cycle regimen (×2 cycles) as previously described (30). Experimental groups (two animals per group) for this study included treatment with (*a*) SPARC, (*b*) SPARC + 5-FU, (*c*) 5-FU, and (*d*) saline (30). All animals received care according to standard animal care protocol and guidelines. Immunostaining was done as previously described (32) with the following primary antibodies: α-SPARC (4 μg/mL) and mouse α-VDR (5 μg/mL).

Statistical Methods
Statistical difference between groups was determined by ANOVA followed by post hoc comparison with Student's t test. $P < 0.05$ was considered statistically significant.

Results

VDR mRNA Is Low in Tumors Resistant to Chemotherapy

We previously generated MIP101 cells resistant to 5-FU, irinotecan (CPT-11), cisplatin, and etoposide, with an IC_{50} at least eight times higher than the parental sensitive cell line (30). A global gene expression analysis using Affymetrix oligonucleotide microarrays revealed a 3-fold decrease in *VDR* levels in all four chemotherapy-resistant MIP101 cell lines (MIP/5-FU, MIP/CPT, MIP/etoposide, and MIP/cisplatin) when compared with the sensitive MIP101 cells. This microarray result was validated by semiquantitative reverse transcription-PCR showing significantly lower mRNA levels of *VDR* in resistant cell lines when compared with the sensitive parental cell line (Fig. 1A). A similar pattern was also observed in another set of sensitive and resistant colorectal cancer cells (HCT 116), where a significant decrease in both *SPARC* and *VDR* expression was observed in chemotherapy-resistant cells (Fig. 1A). The expression of *VDR* could be up-regulated by exposing MIP101 or resistant MIP/5-FU cells to 62.5 nmol/L 1,25-D3 for 24 h (Fig. 1B). From these same microarray analysis results showing lower levels of *VDR* gene expression in therapy-refractory cells, we previously described another gene, *SPARC*, the expression of which was similarly decreased in therapy-refractory cancer cells. This observation was intriguing and led us to wonder (*a*) if up-regulation of VDR by vitamin D also influences the levels of SPARC expression, and (*b*) if there was a correlation between chemotherapy sensitivity and VDR levels. We therefore examined the effect of treating sensitive MIP101 cells and CPT-11–resistant MIP/CPT cells with 62.5 nmol/L 1,25-D3 and noted an increase in *SPARC* levels in both cell types (Fig. 1C). Exposure of MIP/CPT cells to CPT-11

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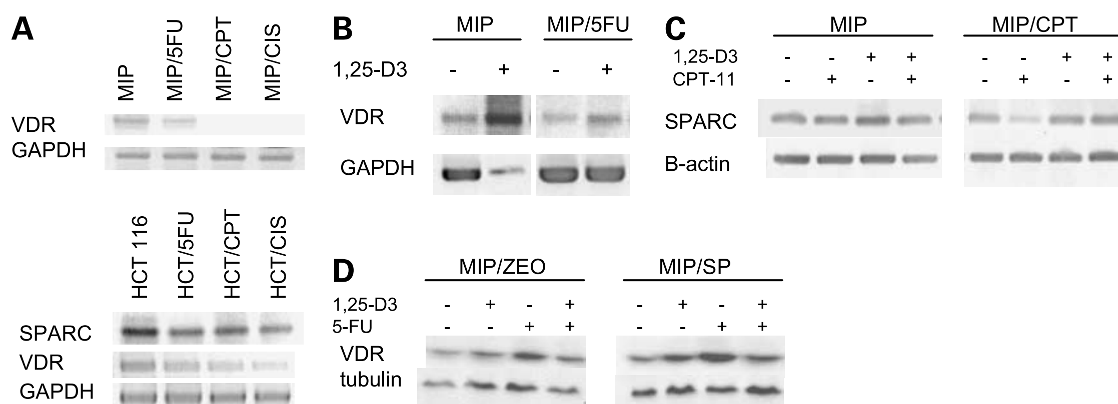

Figure 1. Human VDR and SPARC mRNA and protein levels in colorectal cancer cells sensitive or resistant to chemotherapy. VDR mRNA levels assessed by semiquantitative reverse transcription-PCR in two sets of colorectal cancer sensitive and resistant cells: sensitive MIP101 (*MIP*) and resistant cell lines [MIP/5-FU, MIP/CPT, and MIP/cisplatin (*MIP/CIS*)] and sensitive HCT 116 and resistant cells (HCT/5-FU, HCT/CPT, and HCT/cisplatin; **A**), and following exposure to 62.5 nmol/L 1,25-D3 for 24 h (**B**). **C,** SPARC mRNA expression following exposure to 62.5 nmol/L 1,25-D3 ± 200 μmol/L CPT-11. **D,** VDR protein levels following 24-h exposure with 62.5 nmol/L 1,25-D3 ± 1,000 μmol/L 5-FU.

decreased *SPARC* gene expression, but exposure to 1,25-D3 alone elevated *SPARC* levels that persisted even in the presence of CPT-11. In fact, cells that are more sensitive to chemotherapy and overexpress *SPARC*, as in MIP101 cells stably transfected with *SPARC* (MIP/SP; ref. 30), seem to have higher baseline levels of VDR protein in comparison with their less sensitive parental cell line transfected with the empty vector (MIP/Zeo; Fig. 1D). *VDR* expression increases even more significantly following a 24-h *in vitro* exposure to either 5-FU or 1,25-D3, or their combination, in MIP/SP cells than in controls (Figs. 1D and 2). These results indicate that cancer cells that are resistant to chemotherapy have low levels of both *VDR* and *SPARC*, both of which can be increased following exposure to 1,25-D3. This points to the possibility that increasing both VDR and SPARC expression with 1,25-D3 in colorectal cancer cells may augment their response to chemotherapy.

1,25-D3 Increases Sensitivity of Resistant Colorectal Cancer Cells to Chemotherapy

We wondered about a potential relationship between the levels of SPARC and VDR that could influence cancer cell response to chemotherapy. We next examined whether 1,25-D3 influences the relative sensitivity of colorectal cancer cell lines to chemotherapy and noted that it significantly diminished cell viability in sensitive and resistant cells when concomitantly exposed to chemotherapy in a dose-dependent fashion. Of particular significance is the observation that there was resensitization of the 5-FU–resistant MIP/5-FU cells and CPT-11–resistant MIP/CPT cells to chemotherapy following incubation with 62.5 nmol/L 1,25-D3 (Fig. 3A), whereas lower concentrations of 1,25-D3 had no effect (data not shown). There was no significant effect on cell viability when MIP/5-FU cells were treated with 1,000 μmol/L 5-FU for 24 h (cell viability was 100% in controls and remained unchanged at 110 ± 7.4% following treatment with 5-FU; $P = 0.08$). However, cell viability decreased from 100 ± 5.8% (62.5 nmol/L 1,25-D3 alone) to 88.6 ± 4.0% when exposed to 1,25-D3 and 1,000 μmol/L 5-FU ($P = 0.04$). Similarly, cell viability decreased significantly in CPT-11–resistant MIP101 cells (MIP/CPT) from 112.0 ± 10.9% to 84.9 ± 8.5% ($P = 0.03$) following coincubation with 1,25-D3 and 200 μmol/L CPT-11. We also assessed whether vitamin D_2 (ergocalciferol) had a similar effect on cell viability and noted that incubation with ergocalciferol at much higher concentrations (1 and 2.5 μmol/L) had no effect on cell viability in chemotherapy-resistant

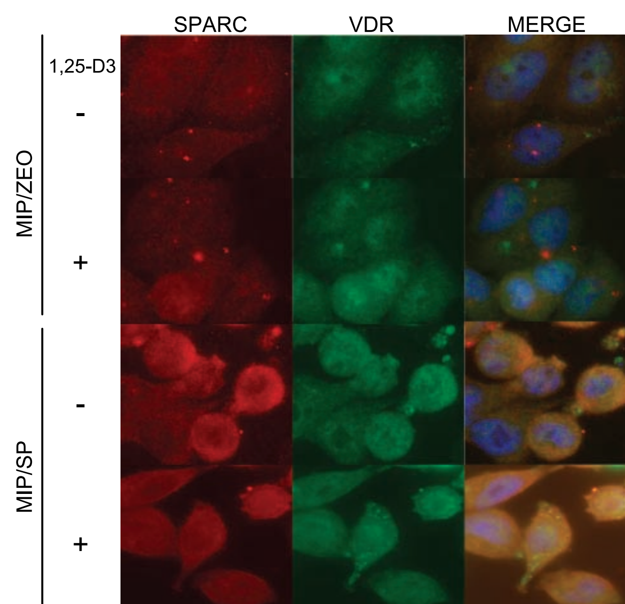

Figure 2. Immunofluorescence staining of SPARC and VDR expression in control MIP101 (MIP/Zeo) and MIP/SP cells following exposure to 2.4 nmol/L 1,25-D3 for 24 h (magnification, ×40).

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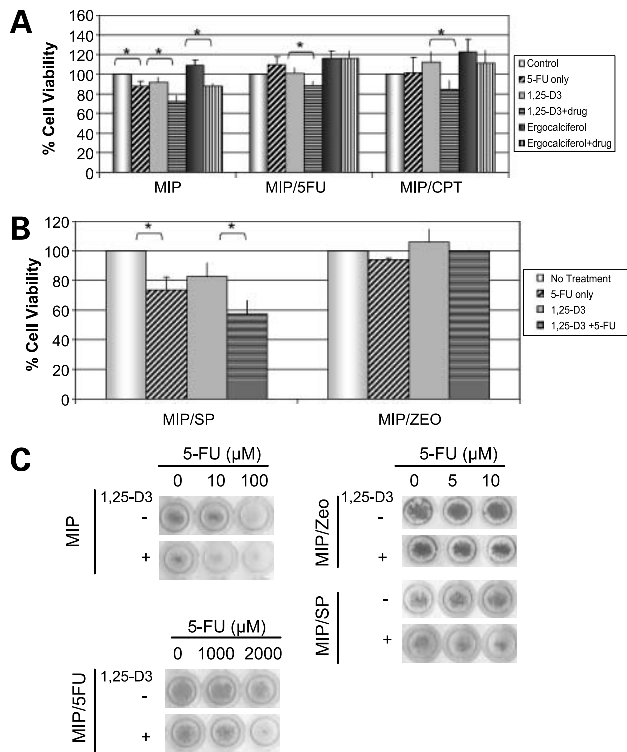

Figure 3. Cell viability of sensitive MIP101, resistant MIP/5-FU, MIP/CPT, and cells overexpressing SPARC (MIP/SP) following exposure to 62.5 nmol/L 1,25-D3 or 2.5 μmol/L ergocalciferol in combination with either 1,000 μmol/L 5-FU or 200 μmol/L CPT-11 (**A**), or to lower concentration (2.4 nmol/L) of 1,25-D3 in combination with 500 μmol/L 5-FU (**B**; $n = 3$). *, $P < 0.05$. **C,** colony-forming assay showing increased sensitivity of MIP101, resistant MIP/5-FU, and sensitive MIP/SP cells following incubation with 1,25-D3 and 5-FU at increasing concentrations.

MIP/5-FU and MIP/CPT cells (Fig. 3A; only 2.5 μmol/L ergocalciferol is represented). Only MIP101 cells seemed to have reduced cell viability when concomitantly exposed to chemotherapy (from 108.5 ± 5.8% to 87.3 ± 2.9%; P = 0.01; Fig. 3A).

Our earlier observation revealed that highly chemotherapy-sensitive, *SPARC*-overexpressing MIP/SP cells had higher levels of *VDR* expression in comparison with the parental cell lines (Fig. 1D). This expression could be further increased following exposure to 1,25-D3. Therefore, we wondered if the higher basal levels of VDR in the MIP/SP cells correlated with greater sensitivity, and whether this chemosensitivity could be further enhanced following exposure to 1,25-D3. This was indeed the case, as cell viability decreased significantly from 100.00 ± 0.01% in MIP/SP cells to 73.3 ± 8.7% (P = 0.006) with only 500 μmol/L 5-FU alone, and even further to 57.5 ± 8.8% (P = 0.03) following exposure to 500 μmol/L 5-FU in combination with only 2.4 nmol/L 1,25-D3 (Fig. 3B). These same experimental conditions with lower 1,25-D3 and 5-FU concentrations had negligible effect on the control of MIP/Zeo cells. This enhanced sensitivity to chemotherapy following concomitant exposure to 1,25-D3 was further supported by the results of the clonogenic assay, where even lower concentrations of 5-FU were required in the presence of 1,25-D3 to produce greater inhibition of cell growth (Fig. 3C).

1,25-D3 Augments Chemotherapy Sensitivity by Increasing Apoptosis through Inactivation of Akt

To understand how 1,25-D3 and SPARC may be interacting to increase chemosensitivity, we next examined if there was an effect on apoptosis. The extent of apoptosis was significantly greater following incubation with only 2.4 nmol/L 1,25-D3, resulting in 7.34 ± 0.97% of MIP/SP cells undergoing apoptosis, which increased to 10.98 ± 1.05% (P = 0.01) in combination with 1,000 μmol/L 5-FU (Fig. 4A). In contrast, cells that express low levels of SPARC (MIP/Zeo cells) had no significant change in the percentage of cells undergoing apoptosis when incubated with 5-FU and this lower concentration of 1,25-D3.

To examine the potential signaling pathway for this enhanced apoptotic response to 1,25-D3 in the presence of higher SPARC levels, we initially assessed the Akt/protein kinase B survival pathway. We noted that MIP/SP cells had lower baseline levels of p-Akt, which decreased even more significantly following incubation with 2.4 nmol/L 1,25-D3, whereas p-Akt levels remained unchanged in MIP/Zeo cells following exposure to this concentration of 1,25-D3 (Fig. 4B). Similarly, levels of Bad were significantly lower after exposure to 1,25-D3 in MIP/SP cells (Fig. 4B). Levels of phosphatase and tensin homologue (PTEN) were unchanged following incubation with either 1,25-D3 or 5-FU.

1,25-D3 Enhances the Delay of Cell Cycle Progression in the Presence of SPARC

1,25-D3 significantly delayed progression through the S phase of the cell cycle in MIP/Zeo and MIP/SP cells (Fig. 5A). Following thymidine synchronization, this failure to progress could be observed as early as 3 h, where 42.4% of the MIP/Zeo cells remained in G_1 phase at 6 h,

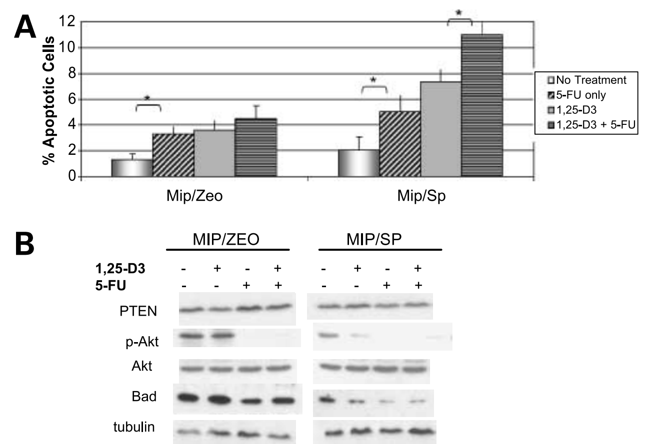

Figure 4. **A,** assessment of apoptosis of control MIP/Zeo and MIP/SP cells following incubation with 2.4 nmol/L 1,25-D3 and 1,000 μmol/L 5-FU for 24 h by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay ($n = 3$). *, $P < 0.05$. **B,** immunoblots; representative of three independent studies.

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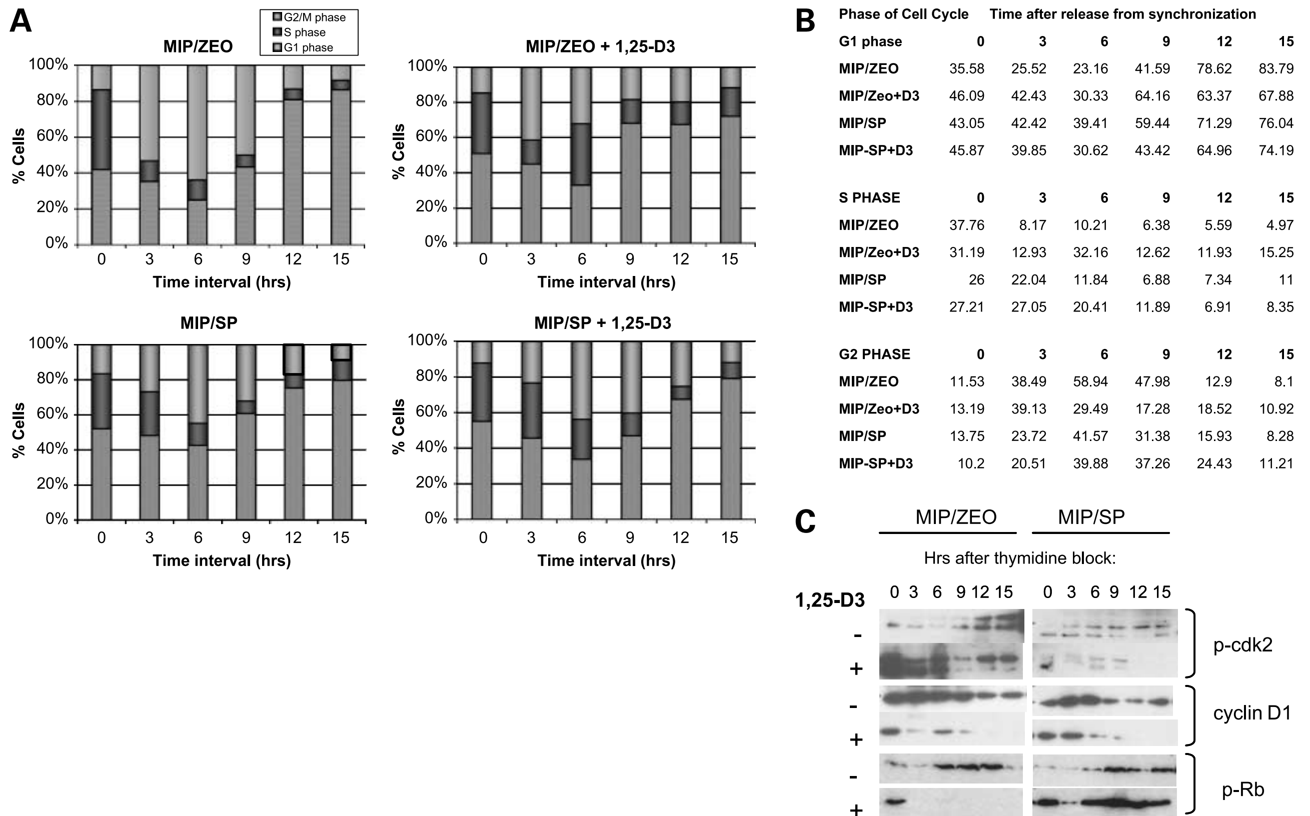

Phase of Cell Cycle	Time after release from synchronization					
G1 phase	0	3	6	9	12	15
MIP/ZEO	35.58	25.52	23.16	41.59	78.62	83.79
MIP/Zeo+D3	46.09	42.43	30.33	64.16	63.37	67.88
MIP/SP	43.05	42.42	39.41	59.44	71.29	76.04
MIP-SP+D3	45.87	39.85	30.62	43.42	64.96	74.19
S PHASE	0	3	6	9	12	15
MIP/ZEO	37.76	8.17	10.21	6.38	5.59	4.97
MIP/Zeo+D3	31.19	12.93	32.16	12.62	11.93	15.25
MIP/SP	26	22.04	11.84	6.88	7.34	11
MIP-SP+D3	27.21	27.05	20.41	11.89	6.91	8.35
G2 PHASE	0	3	6	9	12	15
MIP/ZEO	11.53	38.49	58.94	47.98	12.9	8.1
MIP/Zeo+D3	13.19	39.13	29.49	17.28	18.52	10.92
MIP/SP	13.75	23.72	41.57	31.38	15.93	8.28
MIP-SP+D3	10.2	20.51	39.88	37.26	24.43	11.21

Figure 5. Cell cycle analysis of MIP/Zeo and MIP/SP cells following incubation with 62.5 nmol/L 1,25-D3 after initial synchronization with double thymidine block (**A** and **B**, numerical values within columns represent to cells in each phase of cell cycle). **C,** assessment of cell cycle regulators following incubation with 1,25-D3 (62.5 nmol/L) by immunoblotting.

compared with only 25.5% of untreated cells (Fig. 5B). Similarly, by 6 h, 32.2% of MIP/Zeo cells exposed to 1,25-D3 remained in S phase in comparison with only 10.2% untreated cells. Although a delay to progress from the G_1 phase was seen with MIP/SP, this did not seem to be influenced by 1,25-D3. However, the effect of 1,25-D3 was more apparent in the S phase, as a significant delay could be noted as early as 3 h; by 6 h, 20.4% of MIP/SP cells remained in this phase of the cell cycle following exposure to 1,25-D3, whereas only 11.8% of the untreated MIP/SP cells remained in S phase (Fig. 5B).

This delay in cell cycle progression caused by 1,25-D3, which seemed to be even more pronounced in MIP/SP cells, was influenced by changes in key cell cycle regulators. There was a dramatic down-regulation of cyclin D1 expression in both MIP/Zeo and MIP/SP cells after exposure to 1,25-D3 (Fig. 5C) but an increased phosphorylation of cdk-2 in MIP/Zeo cells in the first 6 h after treatment with 1,25-D3. In MIP/SP cells, exposure to 1,25-D3 resulted in down-regulation of both cyclin D1 and hypophosphorylation of cdk-2 was observed. Interestingly, phosphorylation of the retinoblastoma (Rb) protein was significantly affected and diminished after exposure to 1,25-D3 in MIP/Zeo cells, whereas hyperphosphorylation was observed in MIP/SP cells.

Administration of Exogenous SPARC Increases VDR Expression in Tumor Xenografts *In vivo*

We next assessed VDR expression in tumor xenografts of MIP101 cells harvested from mice that had previously been treated with either saline (control), 5-FU only, exogenous SPARC, or a combination of 5-FU and SPARC (30). Higher VDR protein levels were seen in tumor xenografts harvested from animals treated with either SPARC alone or in combination with 5-FU than in tumors from saline-treated controls (Fig. 6). Tumors from animals treated with 5-FU alone showed lower levels of VDR expression.

Discussion

We previously identified SPARC as a protein that reverses chemotherapy sensitivity in colon cancers from a microarray study of chemotherapy resistance genes (30). From this same analysis, *VDR* was concomitantly underexpressed at the transcriptional level in chemotherapy-refractory colon cancer cells. It has previously been shown that VDR expression is gradually lost during tumor progression (33, 34), even to levels below those detected in normal mucosal epithelium in high-grade carcinomas (35). A more favorable prognosis is observed with higher

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VDR levels (34). Given its growth inhibitory properties, it is not surprising that chemotherapy-resistant colorectal cancer cells favor a state with significantly lower VDR expression.

These observations, taken together with our previous studies showing chemosensitizing properties with SPARC, led us to speculate whether exposure to 1,25-D3, which up-regulates VDR expression in colon cancer, may interact with SPARC to further augment chemotherapy sensitivity. This was indeed the case, as we were able to show that, in the presence of higher levels of SPARC, 1,25-D3 reduced the viability of MIP/SP cells significantly more than in control MIP/Zeo cells. More importantly, the concentration required to achieve this effect was 26-fold lower than that required to reduce cell viability in the control cells (2.4 nmol/L compared with 62.5 nmol/L 1,25-D3) in the presence of half the concentration of 5-FU (500 μmol/L in the presence of SPARC instead of 1,000 μmol/L 5-FU in controls). This concentration of 62.5 nmol/L required to induce growth inhibition in MIP101 cells is within the range observed in other studies. For example, previous reports using HT-29 cells showed that growth inhibition following 1,25-D3 treatment could be achieved with concentrations as low as 10 nmol/L, whereas in other studies, 0.1 to 1.0 μmol/L was required to show a similar effect (19, 22). In MIP101 cells, at least 62.5 nmol/L of 1,25-D3 was required to induce growth inhibition, whereas this significantly decreased to 2.4 nmol/L in the presence of SPARC. Interestingly, a study making a direct comparison between 1,25-D3 and a noncalcemic analogue, paricalcitol, using HT-29 cells, showed that similar doses of both 1,25-D3 and paricalcitol were required to effectively inhibit growth of HT-29 (ED_{50}, 17 nmol/L) *in vitro* (22). As an extension to this observation, and in view of our own results, one would predict that the use of noncalcemic vitamin D analogues in combination with SPARC would have a similar synergistic effect on tumor growth inhibition. Moreover, an even higher dose of the noncalcemic analogue in combination with a much lower dose of chemotherapy could be used to achieve even greater inhibition of tumor growth in the presence of SPARC.

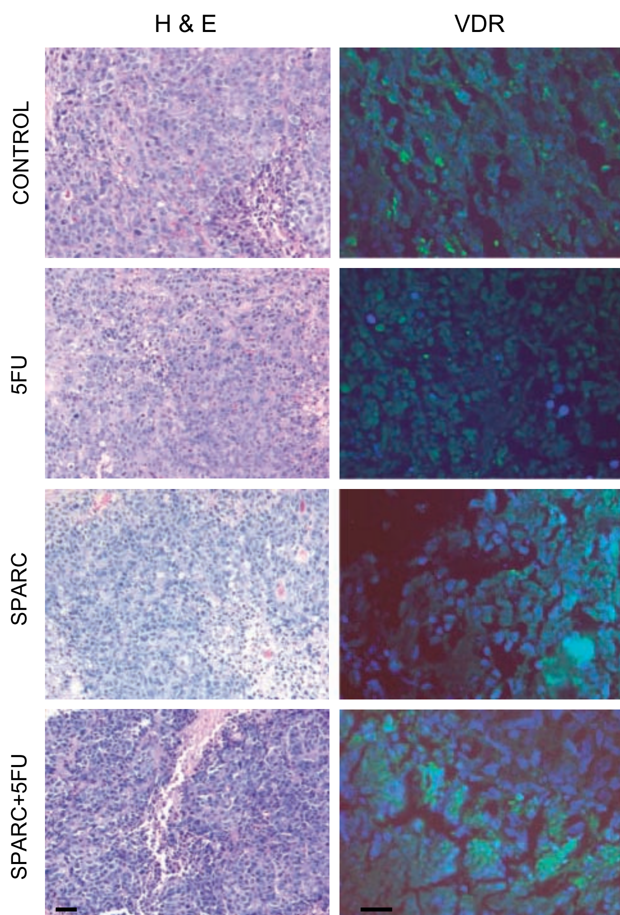

Figure 6. Tumors from mouse xenografts show increased VDR (*green fluorescence*) expression following i.p. administration of SPARC (n = 2 per group; representative sections of 6 μm). Bar, 15 μm. *Blue,* 4′,6-diamidino-2-phenylindole nuclear stain.

SPARC gene expression is low in chemotherapy-resistant MIP/CPT cell lines, but this expression increased following exposure to 1,25-D3, thereby suggesting that VDR may be able to modulate SPARC levels to enhance chemosensitivity in therapy-unresponsive cells. However, in sensitive cells where SPARC expression is higher, there is no further up-regulation of SPARC in response to 1,25-D3 exposure. It would seem that in these chemotherapy-responsive cell lines with higher levels of SPARC, the growth inhibitory effects of 1,25-D3 may be independent of SPARC. Not only does 1,25-D3 up-regulate SPARC expression in chemotherapy-resistant cells but cells overexpressing SPARC (MIP/SP cells) also have higher levels of VDR, which further increase on stimulation with 1,25-D3. This synergistic effect of SPARC and VDR (through 1,25-D3 activation) reduces cell viability in MIP/SP cells by an additional 15.8% following exposure to much lower concentrations of 5-FU. These lower concentrations of 5-FU and 1,25-D3 were ineffective in decreasing cell viability in the absence of higher levels of SPARC, as seen with MIP/Zeo cells. Similarly, a higher percentage of apoptotic cells was observed following concomitant exposure to 5-FU and 1,25-D3 in the presence of higher SPARC levels than in controls. We believe that this is mediated, in part, through inhibition of the survival pathway, Akt, as exposure to 1,25-D3 in MIP/SP cells significantly inhibited the activation of Akt, as noted by the absence of phosphorylated Akt in these cells. This then translated to significantly lower levels of the antiapoptotic protein Bad.

In addition to its effect in promoting apoptosis through inhibition of the Akt survival pathway, we also noted that 1,25-D3 delayed cell cycle progression through the G_1-S phase in both MIP/SP and MIP/Zeo cells, as previously reported (36, 37). Cell cycle progression is tightly regulated by a series of events that include the formation of such complexes as cyclin D-cdk4/cdk6 and cyclin E-cdk2, which are catalytically active during G_1 phase and whose main function is to phosphorylate the Rb protein (36, 37).

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Similar to other studies, we also observed lower cyclin D1 expression following exposure to 1,25-D3 in both MIP/Zeo and MIP/SP. There was also a concomitant decrease in p-cdk2 levels and dephosphorylation of Rb following exposure of MIP/Zeo cells to 1,25-D3. Whereas a similar decrease was observed in p-cdk2 levels in MIP/SP cells in response to 1,25-D3, hyperphosphorylation of Rb was instead observed as a result of exposure to 1,25-D3. Most reports indicate that 1,25-D3–induced effects on cell cycle are associated with dephosphorylation of Rb (37–39). This effect was also observed in our control MIP/Zeo cells exposed to 1,25-D3. Interestingly, hyperphosphorylation of Rb was observed in cells overexpressing SPARC (MIP/SP cells) following exposure to 1,25-D3. This paradoxical observation of hyperphosphorylation of Rb was surprising, and warrants further investigation. We have previously shown that SPARC delays cell cycle progression through the G_1-S phase in MIP101 colorectal cancer cells (30), and a similar effect has also been shown in a variety of other cells (40, 41). Exogenous exposure to SPARC decreases the transcription of cyclin D1 while increasing the levels of $p21^{WAF1}$ (40). $p21^{WAF1}$ is known to suppress the activity of cyclin D1-cdk complexes through dephosphorylation, thereby inhibiting cell proliferation (42). In smooth muscle cells, exogenous SPARC causes cell cycle arrest by reducing cdk-2, p107, and cyclin A levels while hypophosphorylating the Rb protein (43). One possible interpretation of our finding is that cell cycle arrest in MIP/SP cells is not Rb dependent and, therefore, despite the presence of Rb in its hyperphosphorylated state, delayed cell cycle progression following exposure of SPARC-overexpressing cells to 1,25-D3 can still occur. It is possible that other cell cycle regulators may play a greater influence in an environment of SPARC abundance (e.g., other Rb pocket proteins, such as p107, whose levels have been reported to be reduced in the presence of SPARC; ref. 43).

We observed that exogenous administration of SPARC in mice, which we have previously shown to be capable of inducing complete tumor regression in tumor xenograft mouse models (30), also increases VDR protein expression in these tumor xenografts. This suggests that administration of SPARC, which enhances chemosensitivity, also up-regulates VDR, thereby indicating that administration of 1,25-D3 may be efficacious when used in combination with SPARC and chemotherapy. The mechanism by which this occurs requires further investigation.

It is clear that novel strategies are required to enhance therapy sensitivity to chemotherapeutic agents to achieve effective tumor regression. Our previous study showed the chemosensitizing effect of SPARC in therapy-refractory tumors, which resulted in complete tumor regression in mouse xenograft models. This current study now reveals that 1,25-D3, at low concentrations, is effective in augmenting the susceptibility of therapy-refractory cancer cells to chemotherapy in the presence of high levels of SPARC by up-regulating VDR expression. This synergistic effect of SPARC and VDR in abolishing cell proliferation and increasing apoptosis can be achieved at much lower doses of 1,25-D3 and chemotherapy. Therefore, this combination would not only enhance tumor regression but it would also dramatically diminish the side effects that are often associated with the doses of chemotherapy and vitamin D that are currently required to achieve therapeutic response. The synergism between SPARC and 1,25-D3 in enhancing apoptosis and inhibiting cell cycle arrest in colorectal cancer cells may be a useful armamentarium that can be exploited as an adjunct to conventional chemotherapy.

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Synergism between vitamin D and secreted protein acidic and rich in cysteine–induced apoptosis and growth inhibition results in increased susceptibility of therapy-resistant colorectal cancer cells to chemotherapy

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