Improved Response to *nab*-Paclitaxel Compared with Cremophor-Solubilized Paclitaxel is Independent of Secreted Protein Acidic and Rich in Cysteine Expression in Non-Small Cell Lung Cancer

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Background: The secreted protein acidic and rich in cysteine (SPARC) is a matricellular glycoprotein that is produced by tumor and/or neighboring stroma. SPARC expression is thought to facilitate the intracellular accumulation of nanoparticle albumin-bound paclitaxel (*nab*-paclitaxel, abraxane [ABX]). Gene hypermethylation is a common mechanism for loss of SPARC expression in non-small cell lung cancer (NSCLC). We aim to demonstrate the role of SPARC expression as biomarker for treatment selection using ABX in NSCLC and to evaluate the presence of synergistic antitumor effect when a demethylating agent is combined with ABX.

Methods: We analyzed the *SPARC* messenger RNA expression and *SPARC* gene methylation status in 13 NSCLC cell lines and 22 minimally passaged patient-derived (PD) NSCLC tumors using real-time (RT) polymerase chain reaction. The effect of ABX on tumor growth was compared with cremophor-solubilized paclitaxel (taxol) in severe combined immunodeficiency mice bearing SPARC-positive PD xenografts. The effect of pretreatment with a demethylating agent, 5-Aza-2′-deoxycytidine (DEC) in SPARC-negative tumors was assessed.

Results: *SPARC* expression was weak to absent in 62% of established NSCLC cell lines and 68% of PD NSCLC tumor xenografts. *SPARC* expression could be up-regulated/restored by DEC treatment in both SPARC-negative cell lines and PD xenografts in vitro and in vivo. ABX demonstrated better antitumor efficacy than equitoxic dose of taxol in SPARC-expressing xenografts and some SPARC-negative xenografts. At equimolar doses in vitro, there was similar increased cytotoxicity on DEC pretreatment with either ABX or taxol in SPARC-negative cell lines. At equitoxic doses, there was similar additive antitumor activity of DEC with either ABX or taxol in SPARC-negative PD xenografts.

Conclusion: Endogenous SPARC status is somewhat uncorrelated with response to ABX in NSCLC. The greater antitumor effect of ABX compared with equitoxic dose of taxol observed in SPARC-expressing NSCLC tumors can also be seen in some SPARC-negative tumors. DEC pretreatment similarly enhanced antitumor activity with either ABX or taxol in SPARC-negative tumors.

Key Words: *nab*-paclitaxel, Cremophor-solubilized paclitaxel, SPARC, 5-aza-2′-deoxycytidine, Non-small cell lung cancer (NSCLC).

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Secreted protein acidic and rich in cysteine (SPARC)/osteonectin is a nonstructural matricellular calcium-binding glycoprotein. SPARC is involved in physiological cell-matrix interactions such as cell migration, angiogenesis, and tissue remodeling. It is expressed in numerous malignancies such as gastric, colorectal, and head and neck (H and N) cancers.[1,2] SPARC expression is thought to facilitate drug distribution in tumors and enhance clinical effectiveness of nanoparticle albumin-bound paclitaxel (*nab*-paclitaxel, abraxane, designated ABX in this article) because of SPARC-binding characteristics of albumin.[3–7] ABX was developed to avoid cremophor/ethanol-associated toxicities associated with the parent compound (cremophor-solubilized paclitaxel designated as taxol in this article). Moreover, nanoparticle albumin-bound chemotherapy provides a new paradigm for breaching the blood-stroma barrier to reach the tumor cell, by targeting a previously unrecognized tumor-activated albumin-specific biologic pathway with a nanoshell of the human blood protein albumin. This nanoshuttle system activates an albumin-specific (Gp60) receptor-mediated transcytosis path through the cell wall of proliferating tumor endothelial cells using caveolin 1 activated caveolar transport.[8] Once in the stromal microenvironment, the albumin-bound drug is preferentially localized by a second albumin-specific binding protein SPARC, secreted into the stroma by tumor cells. The resulting collapse of stroma surrounding the tumor cell enhances the delivery of the *nab*-chemotherapeutic to the tumor cell itself.

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ABX, a nanoparticle form of paclitaxel, is the first clinical example of this *nab*-driven chemotherapy platform.

SPARC expression seemed to be correlated with antitumor response to ABX in patients with H and N cancer, with SPARC overexpression as determined by immunohistochemistry (IHC) leading to improved response to ABX in tumor models. A study of intraarterial ABX showed a high overall response rate of 78% in 54 patients with H and N cancer.[9] Further characterization of the tumors from responders showed that 91% of patients with H and N cancer responding to ABX had SPARC-positive tumors. SPARC-negative patients exhibited significantly lower response rate of 25% versus the overall response rate of 75% (all patients, SPARC positive and negative). The SPARC-positive tumors had a response rate of 83%.[10] In an in vivo tumor model, stable SPARC-overexpressing lines of PC3 prostate cancer xenograft exhibited enhanced response to ABX relative to wild type (WT) PC3.[11] These lend support to the hypothesis that SPARC expression in tumor tissues can correlate with drug response, and this information can be used to select the appropriate patient population.

In non-small cell lung cancer (NSCLC), SPARC is found to be strongly expressed by IHC in peritumoral stromal fibroblasts.[12] Strong SPARC expression was found in approximately 70% of cases. Although SPARC-expressing cancer cells can be found located adjacent to necrotic areas, SPARC expression in cancer cells per se was typically rare. Gazdar and coworkers[13] demonstrated the lack of *SPARC* expression in 83% of NSCLC cell lines. Aberrant methylation was found in 75% of NSCLC cell lines with restoration of *SPARC* expression after treatment with the demethylating agent 5-Aza-2′-deoxycytidine (DEC). Methylation of *SPARC* gene occurred in 65% of primary lung cancer, resulting in loss of or weak SPARC protein expression in tumor cells by IHC. In a multivariate Cox's proportional hazard model, promoter methylation of *SPARC* was found to be an independent adverse prognostic factor next to tumor stage in adenocarcinoma cases.

The aim of this study is to investigate whether there is a correlation between tumoral SPARC expression and treatment response to ABX in NSCLC in comparison with taxol. That the mechanism of suppression of *SPARC* expression in NSCLC is largely mediated by promoter region methylation is relevant as there are demethylating agents available for clinical use that may potentially increase *SPARC* expression. We, thus, also aim to evaluate the hypothesis that SPARC reexpression in tumor by pretreatment with a demethylating agent DEC will result in improved efficacy of ABX in NSCLC cell lines and primary tumor with low or absent SPARC expression because of promoter gene hypermethylation.

MATERIALS AND METHODS

Cell Lines and Minimally Passaged (Fewer than 10 Passages) Primary Patient-Derived Tumor Specimen

NSCLC cell lines (H226, H358, H441, H522, H661, H727, H1299, H1650, H1703, H2935, H460, H157, and A549) were purchased from American Type Culture Collection (Manassas, VA) and maintained in Roswell Park Memorial Institute medium 1640 with 10% fetal bovine serum (Sigma, St Louis, MO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Cellgro, Manassas, VA) at 37°C in 5% CO_2.

All patient-derived (PD) NSCLC tumor specimens were derived from patients whose surgeries were performed at Roswell Park Cancer Institute (RPCI; Buffalo, NY). Specimens were processed through the Tissue Procurement Facility for pathologic assessment. Surgical samples were obtained with informed consent from patients and under a research protocol approved by the institutional review board and the research ethics committee at RPCI. Samples were examined for presence of malignant/normal areas before transplantation into animals. Animal experiments were approved by the Institutional Animal Care and Use Committee.

Reagents and Drug Treatment with NSCLC Cell Lines In Vitro

ABX was obtained from Abraxis BioScience (Phoenix, AZ). Taxol was purchased from Ben Venue Labs (Bedford, OH). DEC was purchased from Sigma-Aldrich (St. Louis, MO). All the drugs were prepared and aliquoted and stored at −80°C for use within 1 week. For DEC experiments with NSCLC cell lines in vitro, cells were cultured in medium with DEC (5 μM) for 5 days, with medium changes on days 1 and 3. Either ABX or taxol was added to the changed media on day 3. Control cells were only treated with vehicle of 1‰ DMSO in 1 ml of media. Cells were harvested for RNA extraction, *SPARC* expression analysis, or cell death rate assessment on day 5.

Experiments with Tumor Xenografts In Vivo

Experimental studies were carried out using 6- to 8-week-old CB.17 severe combined immunodeficiency (SCID)/SCID with an average body weight ~25 g. H460 (5×10^6 cells) or PD NSCLC xenografts (mouse-to-mouse passage), which were cut into small fragments (2–3 mm in size), were implanted subcutaneously at the flank region. Tumor growth was monitored by periodic visual inspection at the site of implantation, and the dimensions of the xenografts were measured every 2 to 3 days. Tumor volume was calculated using the following formula: $V = \text{LD} \times (\text{SD})^2/2$, where V is the tumor volume, LD is the longest tumor diameter, and SD is the shortest tumor diameter.

To establish a maximally tolerated dose to be used in experiments involving pretreatment with DEC, groups of mice implanted with H460 cell lines were tested at four dose levels of DEC, i.e., 0.75, 1.5, 2, and 4 mg/kg, administered intraperitoneally as a single dose and monitored for viability (weight loss and death). Two mice from each group were killed 48 hours after completing DEC treatment alone and tumor xenografts harvested to affirm *SPARC*-expression status. For combination drug treatment, ABX was administered by tail vein injection at 30 mg/kg/d for 5 consecutive days. Taxol and DEC were used at 13.4 mg/kg/d and 1.5 mg/kg/d, respectively. The doses for ABX and taxol have been previously demonstrated to be equitoxic at these levels.[5] At the end of drug treatment, the mice were killed. Xenografts were harvested and assayed to determine *SPARC* status. All mice experimental protocols were approved by the Institutional Animal Care and Use Committee of RPCI.

Real-Time Polymerase Chain Reaction

Total RNA was extracted from the samples with Trizol reagent (Invitrogen; Carlsbad, CA), and first-strand complementary DNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen). The forward polymerase chain reaction (PCR) amplification primer of *SPARC* was 5′-AAGATCCATGAGAATGAGAAG-3′ (Ex8-S), and the reverse primer was 5′-AAAAGCGGGTGGTGCAATG-3′ (Ex9-AS). Semiquantitative PCR was carried out for 4 minutes at 95°C for initial denaturation, followed by 33 cycles of 94°C for 25 seconds, 56°C for 25 seconds, and 68°C for 40 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control. Quantitative real-time (RT) PCR was done with SYBR GreenER qPCR superMix for ABI PRISM (Invitrogen) using ABI 7300 RT PCR system (Invitrogen), and GAPDH was used as a housekeeping gene for purpose of normalization.

DNA Extraction and Methylation-Specific PCR

Genomic DNA was obtained from cell lines and primary tumors by using DNeasy Blood and Tissue Kit (Qiagen; Valencia, CA) according to the manufacturer's handbook. The DNA methylation pattern in the CpG island of *SPARC* was determined using the method of methylation-specific PCR (MSP) and done with EZ DNA Methylation Kit (Zymo research, Irvine, CA). Primers for the methylated reaction were Sparc-unmsp-F: 5′-TTTTTTAGATTGTTTGGAGAGTG-3′ (sense), Sparc-unmsp-R: 5′-AACTAACAACATAAACAAAAATATC-3′ (antisense), sparc-msp-F: 5′-GAGAGCGCGTTTTGTTTGTC-3′ (sense), and sparc-msp-R: 5′-AACGACGTAAACGAAAA-TATCG-3′ (antisense). PCR amplification was carried out with bisulfite-treated DNA as a template using specific primer sequences for the methylated and unmethylated status of the gene. Water blanks were included with each assay. PCR amplification was carried out for 12 minutes at 94°C for initial denaturation, followed by 40 cycles of 94°C for 30 seconds, 58°C for 25 seconds, and 68°C for 45 seconds. Results were confirmed by repeating the bisulfite treatment and MSP for all samples.

Antibodies and Western Blot

Polyclonal antihuman SPARC antibody was purchased from R&D System (Minneapolis, MN). Western blot analysis was carried out as the standard method. Xenograft lysates were prepared and subjected to immunoblot analysis using 50 μg of cellular protein as described previously.[14] The levels of β-actin (Sigma) were measured as control for equal loading. Immunoblots were developed with enhanced chemiluminescence (Amersham Bioscience, Golden Valley, MN).

Cell Death Rate analysis

NSCLC cells were seeded into 24-well plates and treated with DMSO or agents. Then cells were harvested and stained with trypan blue and viewed under light microscope. Treated cells were harvested and resuspended in trypan blue/phosphate-buffered saline solution. The number of dead/nonviable cells (blue cells) and viable cells (white cells) were counted in representative randomly selected regions, and the ratio of blue cells to white cells is calculated as the cell death rate (%). Each experiment was performed in triplicate at least three times.

Statistical Analysis

Data from in vitro studies are expressed as the mean and standard deviation representing results from at least three independent experiments using cells derived from separate batches of cultures. To describe the observed variability in the in vivo data and test for differences between groups, a multivariate linear model was fit to each dependent variable (tumor volume). All tests were two sided and tested at a 0.05 nominal significance level. SAS version 9.2 statistical software (Cary, NC) was used for all statistical analyses. Difference was considered statistically significant when the calculated p value was less than 0.05.

RESULTS

SPARC Expression and Methylation Status in NSCLC Cell Lines and PD NSCLC Primary Tumors

To evaluate the expression of *SPARC* in NSCLC cell lines and PD NSCLC specimen, *SPARC* expression was examined by RT PCR in 13 NSCLC cell lines and 22 PD NSCLC tumor xenografts. Specimens are considered SPARC positive when *SPARC* expression is 75% or more relative to the expression of the endogenous control, GAPDH. Specimens are considered as SPARC negative if *SPARC* expression is less than 25% to the expression of GAPDH. Levels in-between are considered SPARC intermediate. As shown in Table 1, 8 of 13 (62%) NSCLC cell lines were SPARC negative relative to the expression of internal control GAPDH. Four of 13 (31%) NSCLC cell lines were SPARC positive. For the 22 PD NSCLC xenografts, 15 of them (68%) were SPARC negative; two were SPARC intermediate. The others are SPARC positive. This is concordant with the previous report.[13]

Tumoral SPARC Reexpression with Demethylating Agent

DNA methylation in gene promoter region is the main mechanism to silence gene expression. The loss of SPARC expression in a large proportion of NSCLC specimens may be due to the methylation in the promoter region of *SPARC* gene. To verify this hypothesis, representative samples of NSCLC cell lines or PD xenografts were analyzed. H226, a SPARC-positive cell line, has the highest relative SPARC expression ratio to endogenous control GAPDH (4.469, Table 1). MSP results showed that there was no methylation detected within its promoter (Figure 1). H460, a SPARC-negative cell line (Table 1), is methylated in *SPARC* promoter region, and there is weak PCR signal with the unmethylated promoter region. On treatment with DEC, the *SPARC* expression is up-regulated. This is shown as the increase of unmethylated promoter of *SPARC* in H460 (Figure 1), which manifested as the upregulation of SPARC protein (Figure 2*A*). Similar results were also observed in vitro with NSCLC cell lines (A549, H460, and H157) and in vivo with PD xenografts (NSCLC_16325 and NSCLC_16384) and H460 xeno-

TABLE 1. SPARC Expression Status Analysis in NSCLC Cell Lines and Patient-Derived Primary NSCLC Xenografts

Sample	Detector	Task	Avg Ct	dCt (Target-ENDO)	Relative Expression (1/2)^dCt	SPARC Status
H157	SPARC	Target	21.85	−0.67	1.591073	Positive
	GAPDH	ENDO	22.52			
H226	SPARC	Target	19.56	−2.16	4.469149	Positive
	GAPDH	ENDO	21.72			
H358	SPARC	Target	33.03	10.28	0.000804	Negative
	GAPDH	ENDO	22.75			
H441	SPARC	Target	35.43	11.79	0.000282	Negative
	GAPDH	ENDO	23.64			
H460	SPARC	Target	34.21	11.07	0.000465	Negative
	GAPDH	ENDO	23.14			
H522	SPARC	Target	32.11	6.6	0.010309	Negative
	GAPDH	ENDO	25.51			
H661	SPARC	Target	22.79	−1.29	2.445281	Positive
	GAPDH	ENDO	24.08			
H727	SPARC	Target	31.39	7.82	0.004425	Negative
	GAPDH	ENDO	23.57			
H1299	SPARC	Target	29.06	7.83	0.004395	Negative
	GAPDH	ENDO	21.23			
H1650	SPARC	Target	21.6	−0.25	1.189207	Positive
	GAPDH	ENDO	21.85			
H1703	SPARC	Target	33.11	12	0.000244	Negative
	GAPDH	ENDO	21.11			
H2935	SPARC	Target	26.22	1.77	0.293209	Intermediate
	GAPDH	ENDO	24.45			
A549	SPARC	Target	33.41	12.36	0.00019	Negative
	GAPDH	ENDO	21.06			
NSCLC_16384	SPARC	Target	n/a	n/d	n/d	Negative
	GAPDH	ENDO	26.69			
NSCLC_16325	SPARC	Target	n/a	n/d	n/d	Negative
	GAPDH	ENDO	25.17			
NSCLC_17265	SPARC	Target	31.45	0.49	0.712025	Intermediate
	GAPDH	ENDO	30.96			
NSCLC_16947	SPARC	Target	n/a	n/d	n/d	Negative
	GAPDH	ENDO	27.36			
NSCLC_17228	SPARC	Target	22.58	−3.46	11.00433	Positive
	GAPDH	ENDO	26.05			
NSCLC_17291	SPARC	Target	23.43	0.38	0.768438	Positive
	GAPDH	ENDO	23.05			
NSCLC_17246	SPARC	Target	24.45	−2.64	6.233317	Positive
	GAPDH	ENDO	27.08			
NSCLC_16898	SPARC	Target	31.2	4.89	0.033726	Negative
	GAPDH	ENDO	26.31			
NSCLC_17531	SPARC	Target	24.54	−0.19	1.140764	Positive
	GAPDH	ENDO	24.73			
NSCLC_16591	SPARC	Target	40	3.75	0.074325	Negative
	GAPDH	ENDO	36.25			
NSCLC_16372	SPARC	Target	30.84	0.04	0.972655	Positive
	GAPDH	ENDO	30.8			
NSCLC_15848	SPARC	Target	40	7.6	0.005154	Negative
	GAPDH	ENDO	32.4			
NSCLC_16465	SPARC	Target	40	9.19	0.001712	Negative
	GAPDH	ENDO	30.81			
NSCLC_15946	SPARC	Target	34.82	1.88	0.271684	Intermediate
	GAPDH	ENDO	32.94			

(*Continued*)

TABLE 1. (*Continued*)

Sample	Detector	Task	Avg Ct	dCt (Target-ENDO)	Relative Expression (1/2)^dCt	SPARC Status
NSCLC xenograft 1	SPARC	Target	40	8.84	0.002182	Negative
	GAPDH	ENDO	31.16			
NSCLC xenograft 2	SPARC	Target	38.87	6.54	0.010746	Negative
	GAPDH	ENDO	32.33			
NSCLC xenograft 3	SPARC	Target	40	8.36	0.003044	Negative
	GAPDH	ENDO	31.64			
NSCLC xenograft 4	SPARC	Target	40	6.2	0.013602	Negative
	GAPDH	ENDO	33.8			
NSCLC xenograft 5	SPARC	Target	40	9.88	0.001061	Negative
	GAPDH	ENDO	30.12			
NSCLC xenograft 6	SPARC	Target	40	7.64	0.005013	Negative
	GAPDH	ENDO	32.36			
NSCLC xenograft 7	SPARC	Target	40	5.73	0.018841	Negative
	GAPDH	ENDO	34.27			
NSCLC xenograft 8	SPARC	Target	40	8.39	0.002981	Negative
	GAPDH	ENDO	31.61			

SPARC, secreted protein acidic and rich in cysteine; NSCLC, non-small cell lung cancer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ENDO, endogenous control; NA, not applicable.

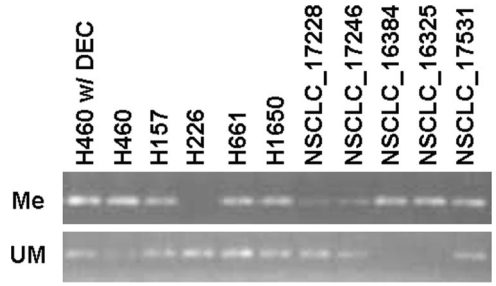

FIGURE 1. Representative examples of methylation-specific PCR assay for *SPARC* in NSCLC cell lines and NSCLC xenografts. H460 cells were treated with DEC (5 μM) in vitro for 3 days. PCR products were visualized on 1.5% agarose gels. w/, treated with; Me, methylated; UM, unmethylated; PCR, polymerase chain reaction; SPARC, secreted protein acidic and rich in cysteine; NSCLC, non-small cell lung cancer; DEC, 5-Aza-2′-deoxycytidine.

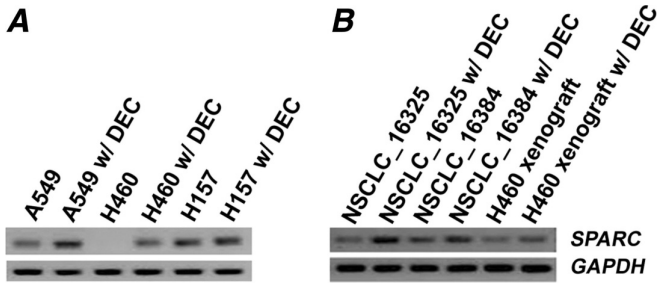

FIGURE 2. Western blot showing that *SPARC* expression in xenografts and NSCLC cell lines is up-regulated on treatment with DEC. *A,* NSCLC cell lines A549, H460, and H157 were treated with 5 μM DEC for 3 days in vitro and then harvested for RT-PCR. *B,* PD NSCLC xenografts and H460 xenografts treated with or without DEC 1.5 mg/kg/d, and xenografts were harvested for SPARC expression analysis. *GAPDH* were used as the endogenous control. w/, treated with; *SPARC*, secreted protein acidic and rich in cysteine; PD NSCLC, patient-derived non-small cell lung cancer; DEC, 5-Aza-2′-deoxycytidine; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, real-time polymerase chain reaction.

graft (Figures 1 and 2). The loss of SPARC expression in NSCLC specimens is largely attributed to the methylation in the promoter region of *SPARC* gene. Our results show that *SPARC* expression can be up-regulated by exposure to DEC in vitro or in vivo.

Endogenous Tumor SPARC Expression and Response to ABX In Vivo

SPARC expression in tumor has been shown to facilitate the transport of albumin-bound drugs.[11] ABX is an albumin-bound paclitaxel. We, thus, sought to compare the antitumor efficacy of ABX with equitoxic dose of taxol in a series of PD NSCLC xenografts with different SPARC expression status. NSCLC_16372 is a SPARC-positive tumor. As shown in Figure 3*A*, the growth of NSCLC_16372 xenograft was suppressed on treatment with either taxol and ABX compared with vehicle control. Nevertheless, ABX was more effective than taxol in inhibiting xenograft growth ($p = 0.0239$). Similar results were observed in NSCLC_15946, a SPARC-intermediate specimen. The tumor growth was significantly inhibited with either taxol ($p = 0.0004$) or ABX ($p < 0.0001$) compared with vehicle control. Again, exposure to ABX displayed a more profound growth suppression than taxol ($p < 0.0001$; Figure 3*B*). NSCLC_16465 and NSCLC_16591 are SPARC-negative tumors. Administration of neither taxol nor ABX showed any effect on tumor growth compared with vehicle control in NSCLC_16,591 ($p = 0.3628$ of taxol to vehicle; $p = 0.1826$ of ABX to vehicle;

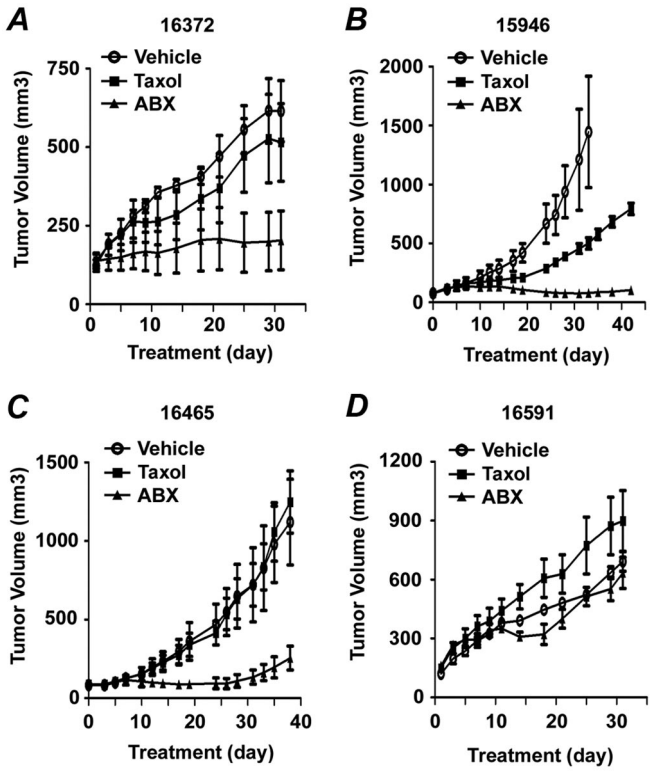

FIGURE 3. Efficacy of ABX compared with taxol in PD NSCLC xenografts. SCID mice bearing SPARC-positive xenografts (NSCLC_16372) (*A*), SPARC-intermediate xenografts (NSCLC_15946) (*B*), or SPARC-negative xenografts, NSCLC_16465 (*C*) and NSCLC_16591 (*D*), were treated with vehicle, equitoxic dose of taxol (13.4 mg/kg), or ABX (30 mg/kg). The overall antitumor efficacy of drugs was measured as tumor volumes every 2 to 3 days. The error bars represented the standard error of the mean. PD NSCLC, patient-derived non-small cell lung cancer; SCID, severe combined immunodeficiency; SPARC, secreted protein acidic and rich in cysteine.

Figure 3*D*). Interestingly, in NSCLC_16465 xenografts, ABX demonstrated significantly greater antitumor effect compared with taxol ($p < 0.0001$), which showed no difference compared with vehicle control. ABX is, thus, generally more effective than taxol at equitoxic doses in inhibiting the growth of PD NSCLC xenografts, and this effect is not consistently correlated with tumor SPARC expression status.

Tumor SPARC Reexpression/Upregulation and Response to ABX or Taxol

Our data indicated that *SPARC* expression was upregulated on treatment with DEC in vitro and in vivo. To establish a tolerable dose to be used in experiments in vivo, groups of mice implanted with H460 cell lines were tested at four dose levels of DEC, i.e., 0.75, 1.5, 2, and 4 mg/kg, respectively. This is based on prior publication showing that DEC administered twice daily intraperitoneally for 5 days was toxic at 10 mg/kg/dose, and 2 mg/kg dose was associated with mild toxicity.[15] There is dose-dependent reexpression of SPARC in H460 xenografts on treatment with DEC. Nevertheless, DEC at 2 and 4 mg/kg was toxic with 6/8 (75%) mice dead between 8 and 12 days after only one DEC dose (without ABX treatment). DEC dose at 1.5 mg/kg was subsequently used in the combination experiments.

To further elucidate the effect of SPARC expression status on the differential antitumor efficacy of ABX in comparison with equitoxic dose of taxol in vivo, experiments in two SPARC-negative PD NSCLC primary xenografts (NSCLC_16325 or NSCLC_16384) and one SPARC negative established NSCLC cell line (H460) were performed. First, SPARC expression in xenografts was up-regulated on exposure to DEC (1.5 mg/kg; Figure 2*B*). Second, compared with vehicle, on treatment with either ABX or taxol alone, only administration with ABX in NSCLC_16325 showed significant antitumor efficacy ($p = 0.0065$). There was, however, no significant difference between ABX compared with taxol in suppressing the growth of these SPARC-negative PD xenografts ($p = 0.4577$ in NSCLC_16325 and $p = 0.9897$ of NSCLC_16384; Figures 4*A*, *B*). Third, we found that the antitumor activities of both taxol and ABX were enhanced to a similar degree by pretreatment with DEC; however, this seeming additive activity did not reach statistical significance ($p = 0.1374$ of ABX + DEC versus ABX and $p = 0.4713$ of taxol + DEC versus taxol in 16,325; $p = 0.8844$ of ABX + DEC versus ABX and $p = 0.7410$ of taxol + DEC versus

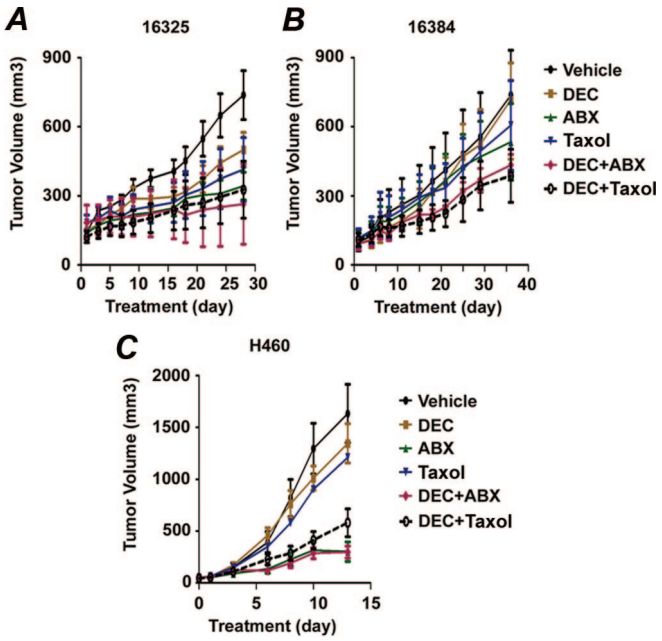

FIGURE 4. Enhanced antitumor efficacy of taxol and ABX by pretreatment with DEC in SPARC-negative xenografts. *A*–*C*, SCID mice bearing SPARC-negative xenografts, NSCLC_16325 (*A*), NSCLC_16384 (*B*), or H460 (*C*), were administered with DEC (1.5 mg/kg), taxol (13.4 mg/kg) or ABX (30 mg/kg) alone or the combination of DEC and taxol or DEC and ABX. The overall antitumor efficacy of drugs was measured as tumor volumes every 2 to 3 days. The error bars represented the standard error of the mean. DEC, 5-Aza-2′-deoxycytidine; SPARC, secreted protein acidic and rich in cysteine; NSCLC, non-small cell lung cancer.

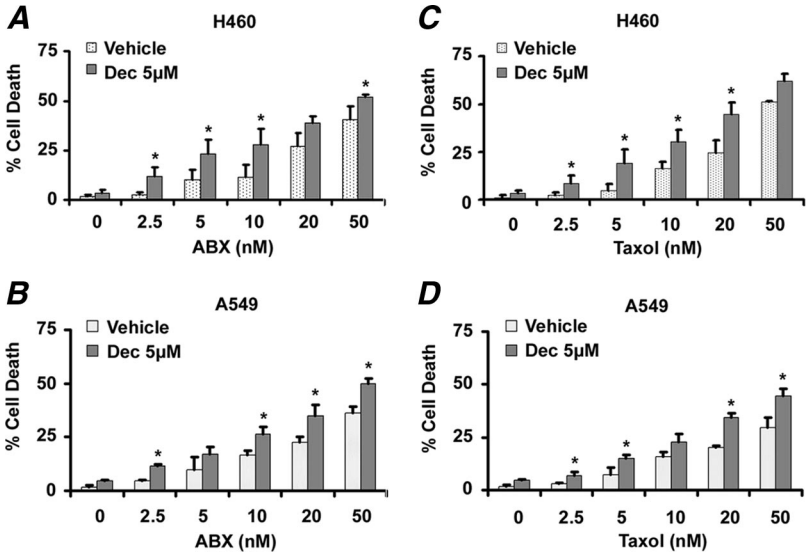

FIGURE 5. Cell death rate analysis with NSCLC cell lines A549 and H460 in vitro showing additive antitumor activity of ABX or taxol by pretreatment with DEC. *A* and *C*, results of H460 cells treated with escalating concentrations of ABX or taxol after DEC 5μM pretreatment. *B* and *D*, results of A549 cells treated with escalating concentrations of ABX or taxol after DEC 5 μM pretreatment. Treated cells were harvested for cell death rates assay (*$p <$ 0.05, compared with ABX or taxol treatment alone). Data represents mean ± standard deviation of three independent experiments. NSCLC, non-small cell lung cancer; DEC, 5-Aza-2′-deoxycytidine.

taxol in 16,384; Figures 4*A*, *B*). Finally, in H460 xenografts, ABX showed superior growth-suppressing activity compared with vehicle or taxol ($p = 0.0002$). Nevertheless, DEC pretreatment resulted in minimal change to ABX antitumor activity, whereas a synergistic effect was seen in combination with taxol ($p = 0.0097$). Nonetheless, the tumor growth inhibition achieved by ABX alone seemed greater compared with DEC with equitoxic dose of taxol, though not statistically significant (84% of ABX versus 66% of DEC + taxol, $p = 0.5402$) (Figure 4*C*).

Pretreatment with DEC to enhance antitumor efficacy of taxol or ABX was also verified in SPARC-negative NSCLC cell lines A549 and H460 in vitro. As shown in Figure 5, DEC enhanced the cytotoxicity of both taxol and ABX in a wide range of doses (2.5, 5, 10, 20, and 50 nM). There is no obvious difference in the antitumor activity between taxol and ABX at equimolar doses.

DISCUSSION

The role of *SPARC* in tumor development seemed to be tumor-type dependent. In H and N cancer, glioma, and melanoma, increased expression of SPARC is correlated with tumor growth and poor survival in these patients.[16–18] Nevertheless, in NSCLC, colon cancer, and ovarian cancer, the expression of SPARC is usually repressed by hypermethylation in its promoter region, which is related to poor patient outcome, with induction of its expression exhibiting antiproliferative effect in some models.[13,19,20] In our study, we affirmed prior results from other investigators that SPARC expression is weak or absent in majority of NSCLC tumors and that a primary mechanism for this is mediated by promoter methylation. These findings are relevant as there are demethylating agents available for clinical use, and there are data suggesting that ABX is an agent that may potentially be targeted toward tumors that over-express SPARC.

To elucidate the role of SPARC status with respect to the antitumor efficacy of ABX specifically in NSCLC, we compared the effect of ABX and taxol in inhibiting tumor growth of SPARC intermediate to positive PD xenografts in vivo. Our data suggest that at equitoxic doses, ABX seems to result in better antitumor response in this subgroup, supporting the initial hypothesis of SPARC expression as a biomarker comparative efficacy between ABX and taxol. These results are compatible with the findings of markedly improved tumor response to ABX in patients with H and N cancer whose tumors overexpress SPARC.[9,10] Moreover, the effect is sustained, and mice in this group treated with ABX had longer viability compared with either taxol or vehicle. Interestingly, further experiments reveal that ABX also showed superior antitumor activity compared with equitoxic dose of taxol in some SPARC-negative NSCLC cells as well. Although β-tubulin III overexpression has been implicated in taxane resistance, the β-tubulin III expression profile in our PD xenografts does not support correlation with sensitivity or resistance to ABX or taxol in our results (experiment not shown).

We also demonstrated that DEC up-regulated the expression of *SPARC* in established NSCLC cell lines and in PD primary tumor xenografts both in vitro and in vivo. Contrary to what is expected, our in vitro data showed that additive antitumor efficacy can be seen in vitro to a similar degree with either ABX or taxol at equimolar doses on pretreatment with DEC at a variety of dose combinations. This is somewhat recapitulated in the in vivo experiments done in PD xenografts wherein the antitumor activity of equitoxic doses of taxol and ABX were both enhanced on pretreatment with DEC to a similar magnitude as well. This is not surprising as the effect of DEC as a demethylating agent can lead to broad changes in expression patterns of various genes apart from *SPARC*. Moreover, there are other factors that can mediate taxane sensitivity, and the results seen with H460 xenografts and in one of the SPARC-negative xenografts (NSCLC_16465) may simply reflect dose response to higher molar dose of ABX when it is equitoxic to taxol in tumors that are innately taxane sensitive. SPARC expression in murine peritumoral tissue may have also con-

tributed to greater ABX activity in some SPARC-negative xenograft experiments. This may also explain the significant antitumor response to ABX alone in H460 xenograft, which would have rendered superfluous any possible contributory effect of DEC otherwise at the ABX dose used for this experiment. Our results thus suggest that the effect of DEC on other proteins may result in additive effects with either ABX or taxol through non-SPARC mechanisms. Given the fact that DEC is FDA-approved drug and ABX is also widely used clinically, the combination treatment of DEC and ABX may be a promising therapeutic strategy for NSCLC.

We acknowledge that our study is limited by the small sample size of unique PD xenografts that were tested. Our experiments were hampered by the protracted time required for establishing the xenografts and lack of growth (i.e., xenografts unable to be established) for certain samples. As indicated earlier, whether SPARC expression in the murine peritumoral tissue could have contributed to the effects seen in the experiments conducted in SPARC-negative tumor samples is not known. Nonetheless, our study showed that a superior antitumor response to ABX relative to equitoxic dose of taxol in PD NSCLC xenografts can be seen in both SPARC-expressing tumors and some SPARC-negative tumors. Additive effects with decitabine pretreatment, using either taxol or ABX are likely mediated by non-SPARC mechanisms. These results need to be verified in a study involving larger numbers of patient samples.

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