Analogs of Vitamin E Epitomized by α-Tocopheryl Succinate for Pancreatic Cancer Treatment

In Vitro Results Induce Caution for In Vivo Applications

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Objectives: α-Tocopheryl succinate (α-TOS) is thought to be toxic only for cancer cells. We ascertained in vitro α-TOS effects on pancreatic cancer (PC) and normal cell growth and verified whether the combination of nontoxic α-TOS and 5-fluorouracil (5-FU) doses causes cancer cell death and whether α-TOS effects are mediated by the proapoptotic proteins Bax/Bak and/or *SMAD4/DPC4* status.

Methods: Five PC cell lines, myoblasts, normal monocytes, wild-type (WT) and Bax/Bak double knockout mouse embryonic fibroblast (MEF) cells, and permanently *SMAD4/DPC4*-transfected PSN1 cells were cultured in 1% and 10% fetal calf serums (FCSs), without or with α-TOS (5–500 μmol/L). Nontoxic 5-FU (0.0001 mmol/L) and α-TOS alone or in combination were also evaluated.

Results: Only PSN1 PC cell line, which had *SMAD4/DPC4* homozygous deletion, was sensitive to nontoxic α-TOS doses (5 μmol/L in 1% FCS and 50 μmol/L in 10% FCS). A 20-μmol/L α-TOS inhibited MEF-WT, not MEF–double knockout growth. Only PSN1 cells were sensitive to nontoxic 5-FU and α-TOS combination. *SMAD4/DPC4* transfection restored PSN1 resistance to the effects of combined 5-FU and α-TOS effects.

Conclusions: Only a minority of PC cells are sensitive to the antiproliferative effects of α-TOS, any sensitivity appearing to be correlated with *SMAD4/DPC4* homozygous deletion and Bax/Bak expression.

Key Words: pancreatic cancer, α-tocopheryl succinate, 5-fluorouracil, Bax/Bak, *SMAD/DPC4*, CAPAN1, BxPC3, MIA PaCa2, PSN1, PANC1, pancreatic cancer cell lines

(*Pancreas* 2010;39: 662–668)

In the last 3 decades, advances in screening, perioperative care, chemotherapy, and radiotherapy have led to a decrease in the expected number of deaths from most cancer types.[1] This progress, however, has not been made for pancreatic cancer, which remains largely incurable: it is estimated that in the United States in the year 2009, this tumor will be diagnosed in 42,470 people, and 35,240 of them will die of the disease.[1]

Currently, the only potentially curative treatment available for pancreatic cancer is a combination of complete tumor resection and adjuvant chemotherapy,[2,3] remaining the 5-year survival rate only 20%.[4,5] This dismal clinical reality depends in part on the high resistance of pancreatic cancer to almost all available antineoplastic drugs, although the results of recent randomized controlled trials and meta-analysis have demonstrated that postoperative gemcitabine significantly delays the development of recurrence and prolongs the overall median survival.[6–8] Equally promising findings have been made after the administration of adjuvant 5-fluorouracil (5-FU) and folinic acid.[9]

The high intrinsic resistance of pancreatic cells to drugs depends on a multifactorial process involving the activation of protooncogenes and/or inactivation of tumor suppressor genes.[10,11] Protooncogene *k-ras* activation and *p53* inactivation, frequently found in pancreatic cancer, may deregulate the transcription of the *MDR1* gene.[12] Another frequent molecular alteration occurring in pancreatic cancer cells is the loss of *SMAD4/DPC4*, which encodes a transcriptional regulator of the transforming growth factor β (TGF-β) signaling cascade.[10] This transcriptional factor participates in the regulation of apoptosis not only by influencing the B-cell lymphoma 2 (Bcl-2)/Bax balance[13] but also by interacting with mutant p53.[14]

Scientific interest is now focused upon an increasing number of anticancer drugs able to induce cell death by targeting mitochondria. These agents, referred to as mitocans, destabilize mitochondria, causing the cytosolic release of apoptogenic factors,[15,16] and have been suggested to enhance the antitumor immune response.[17] Mitocans include vitamin E redox-silent analogs, epitomized by α-tocopherol or α-tocopheryl succinate (α-TOS).[16,18] It has, in particular, been demonstrated that α-TOS has a strong selective proapoptogenic activity against malignant cells[16,18]; the therapeutic effects of α-TOS have also been documented in vitro in several tumor types, including cancer of the prostate,[19] breast,[20] stomach,[21] neuroblastoma,[22] osteosarcoma,[23] and renal cell carcinoma.[24] The antitumoral activity of this compound depends on its ability to target several intracellular pathways, and it can induce cell cycle arrest by reducing the expression of several critical cyclins and cyclin-dependent kinases[17]; it also induces an accumulation of reactive oxygen species in cancer cells, resulting in the activation of downstream proapoptotic signaling pathways.[15] The induction of apoptosis by α-TOS depends on its ability to block Bak BH3 binding to Bcl–extra large (Bcl-x_L) and Bcl-2,[19] the depletion of which leads to the destabilization of mitochondria and the release of cytochrome *c* into the cytosol.[25,26] The newly available cytochrome *c* triggers the activation of Caspase 3, the so-called point-of-no-return in the apoptotic pathway.[25,26]

Only few studies have investigated the cytotoxic effect of α-TOS on pancreatic cancer cells providing contradictory results.[27,28]

The present in vitro study was therefore conducted to investigate whether α-TOS exerts its putative antitumoral effect on pancreatic cancer cells and to ascertain whether α-TOS enhances pancreatic cancer cell sensitivity to 5-FU. The roles of

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Received for publication July 20, 2009; accepted October 30, 2009.
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This work was supported by Ministero Università e Ricerca (Cofin 2005060715_002), Rome, Italy.

SMAD4 and Bak/Bax in mediating the antitumoral effects of α-TOS were also analyzed.

MATERIALS AND METHODS

Cell Lines

The 5 pancreatic cancer cell lines used were BxPC3 (kindly donated by Dr Andrea Galli, University of Florence, Florence, Italy), CAPAN1 and MIA PaCa2 (American Type Culture Collection, Manassas, Va), PANC1 and PSN1 (kindly donated by Prof Aldo Scarpa, University of Verona, Verona, Italy). The cells were kept in continuous culture in conditions described by us elsewhere.[29] Two control cell lines were used: C_2C_{12} myoblasts (kindly donated by Prof Gerolamo Lanfranchi, University of Padova, Padova, Italy) and monocytes obtained from blood donor buffy coats. C_2C_{12} were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, San Giuliano Milanese, Italy) supplemented with 10% fetal calf serum (FCS), 2% L-glutamine, and 0.1% gentamycin (Gibco/BRL, Gaithersburg, Md). Monocytes were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FCS, 1% L-glutamine, and 0.1% gentamycin. The wild-type (WT) and Bax/Bak double knockout (DKO) mouse embryonic fibroblast (MEF) cells (kindly donated by Dr Luca Scorrano, University of Padova) were maintained in DMEM supplemented with 15% FCS, 2% L-glutamine, and 0.1% gentamycin.

SMAD4/DPC4 Plasmid Expression Vector

The expression vector used, pBK-cytomegalovirus (CMV)-*SMAD4/DPC4* (kindly donated by Prof Stephan A. Hahn, University of Bochum, Bochum, Germany), which allows *SMAD4/DPC4* gene expression under CMV promoter control, was subcloned in *Escherichia coli* DH5α (Invitrogen). Plasmid DNA was purified using the High Pure PCR Template Preparation Kit (Roche, Monza, Italy). The vector contained the neomycin-resistance cassette (Neo), which allows the selection of stably transfected eukaryotic cells using the neomycin analog, G418.

PSN1 Cell Line Transfection

Four-microgram plasmid pBK-*CMV-SMAD4/DPC4* expression vectors were incubated with 10-μL Lipofectamine 2000 (Invitrogen) in 500-μL serum and antibiotic-free DMEM for 20 minutes at 25°C, after which the reagent was directly added to each PSN1 cell culture well (200,000 cells) in a 6-well plate format. After 6 hours, media were replaced with fresh serum supplemented media and the cells maintained at 37°C for the subsequent 24 hours. A stably transformed PSN1 cell line was obtained using G418 (Invitrogen) selection (1 mg/mL for 15 days). The surviving colonies were subcultured and established as a permanent transfected PSN1 cell line (PSN1-*SMAD4/DPC4*+). A PSN1-*SMAD4/DPC4*+ cellular suspension was diluted to produce monoclonal colonies, 10 clones being derived and maintained in G418 (1 mg/mL).

Detection of *SMAD4/DPC4* Gene Expression by Reverse Transcription Polymerase Chain Reaction

Three micrograms of total RNA (High Pure RNA Isolation Kit; Roche, Mannheim, Germany) was obtained from 1×10^6 PSN1, PSN1-SMAD4/DPC4+, and MIA PaCa2 cells. Total RNA was reverse transcribed by using the SuperScript II (Invitrogen) and random primers (Invitrogen). The *SMAD4/DPC4* gene was then amplified from 2-μL complementary DNA into a final volume of 25 μL, containing 1.5-mmol/L $MgCl_2$, 200-μmol/L deoxyribonucleotide triphosphate each, 1.25 UTaq Gold DNA polymerase (Applied Biosystems, Monza, Italy), and 0.5-μmol/L forward (5′-CCCAGGATCAGTAGGTGGAA-3′) and reverse (5′-AAGGTTGTGGGTCTGCAATC-3′) primers. The 239-base pair amplicons were analyzed by agarose gel electrophoresis.

TABLE 1. α-Tocopheryl Succinate Effects on Growth of Pancreatic Cancer Cells Cultured in RPMI With 10% FCS (Control) and With the Addition of Increasing Amounts of α-TOS

	Mean ± SD				
	BxPC3	**CAPAN1**	**MIA PaCa2**	**PANC1**	**PSN1**
Control (n = 32)	1.90 ± 0.63	1.59 ± 0.24	1.91 ± 0.48	1.29 ± 0.35	1.63 ± 0.29
Ethanol control (n = 52)	1.56 ± 0.40	1.57 ± 0.40	1.94 ± 0.36	1.31 ± 0.25	1.38 ± 0.41
α-TOS					
5 μmol/L (n = 16)	1.77 ± 0.13	1.58 ± 0.23	2.01 ± 0.49	1.16 ± 0.20	1.54 ± 0.23
10 μmol/L (n = 16)	1.66 ± 0.24	1.59 ± 0.25	1.97 ± 0.48	1.21 ± 0.25	1.48 ± 0.23
20 μmol/L (n = 16)	1.89 ± 0.25	1.66 ± 0.28	1.95 ± 0.44	1.22 ± 0.28	1.44 ± 0.26
50 μmol/L (n = 16)	1.68 ± 0.42	1.58 ± 0.29	1.76 ± 0.58	1.25 ± 0.22	1.26 ± 0.24*
100 μmol/L (n = 16)	1.10 ± 0.45†	1.18 ± 0.14†	0.51 ± 0.21†	0.91 ± 0.24‡	0.40 ± 0.07†
200 μmol/L (n = 16)	0.25 ± 0.04§	0.26 ± 0.03§	0.24 ± 0.02†	0.28 ± 0.03§	0.24 ± 0.01§
500 μmol/L (n = 16)	0.29 ± 0.03§	0.30 ± 0.03§	0.29 ± 0.03†	0.29 ± 0.02§	0.30 ± 0.02§
Test for within-subject effect	F = 56.97; $P < 0.0001$	F = 57.8; $P < 0.0001$	F = 56.20; $P < 0.0001$	F = 31.50; $P < 0.0001$	F = 67.00; $P < 0.0001$
Test for between-subject effect	F = 68.96; $P < 0.0001$	F = 100.7; $P < 0.0001$	F = 47.5; $P < 0.0001$	F = 52.4; $P < 0.0001$	F = 126.3; $P < 0.0001$

Because α-TOS was dissolved in 1% (vol/vol) ethanol, an ethanol control was always run in parallel. Cell growth was assessed by XTT assay before and 24, 48, and 72 hours after treatment. Results at the 72nd hour are reported as mean value ± SD of the $Absorbance450_{nm}$ obtained from a minimum of 16 to a maximum of 52 experimental wells (in brackets), from 4 to 6 separate experimental sets. Findings made at statistical analysis (repeated-measures analysis of variance) are also shown.

*$P < 0.05$ with respect to control; ethanol control; and 5-, 10-, and 20-μmol/L α-TOS.

†$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, 20-, and 50-μmol/L α-TOS.

‡$P < 0.05$ with respect to control and ethanol control.

§$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, 20-, 50-, and 100-μmol/L α-TOS.

TABLE 2. Effects of α-TOS on Growth of Nonneoplastic Cells (C_2C_{12} Myoblasts or Normal Monocytes From Blood Donors) Cultured in RPMI With 1% or 10% FCS (Controls) With the Addition of Increasing Amounts of α-TOS

	C2C12 Myoblasts, Mean ± SD		Monocytes From Blood Donors, Mean ± SD	
	1% FCS	10% FCS	1% FCS	10% FCS
Control (n = 24)	0.94 ± 0.16	1.93 ± 0.13	0.91 ± 0.63	0.89 ± 0.39
Ethanol control (n = 24)	0.95 ± 0.23	2.17 ± 0.38	0.89 ± 0.46	0.75 ± 0.39
α-TOS				
5 μmol/L (n = 24)	0.82 ± 0.15	1.93 ± 0.12	0.76 ± 0.46	0.76 ± 0.44
10 μmol/L (n = 24)	0.84 ± 0.26	1.96 ± 0.13	0.71 ± 0.44	0.73 ± 0.50
20 μmol/L (n = 24)	0.73 ± 0.21	1.96 ± 0.13	0.68 ± 0.48	0.72 ± 0.49
50 μmol/L (n = 24)	0.24 ± 0.11*	1.77 ± 0.16	0.59 ± 0.43	0.70 ± 0.45
100 μmol/L (n = 24)	0.19 ± 0.01*	1.28 ± 0.48†	0.21 ± 0.02‡	0.71 ± 0.35
200 μmol/L (n = 24)	0.22 ± 0.01*	0.41 ± 0.24§	0.23 ± 0.05‡	0.24 ± 0.02§
500 μmol/L (n = 24)	0.34 ± 0.04*	0.23 ± 0.02‖	0.45 ± 0.10	0.32 ± 0.03§
Test for within-subject effect	F = 59.80; $P < 0.0001$	F = 138.40; $P < 0.0001$	F = 6.90; $P < 0.0001$	F = 5.8; $P < 0.0001$
Test for between-subject effect	F = 225.6; $P < 0.0001$	F = 346.4; $P < 0.0001$	F = 4.42; $P < 0.0001$	F = 4.6; $P < 0.0001$

Because α-TOS was dissolved in 1% (vol/vol) ethanol, an ethanol control was always run in parallel. Cell growth was assessed by XTT assay before and 24, 48, and 72 hours after treatment. Results at the 72nd hour are reported as mean value ± SD of the $Absorbance450_{nm}$ obtained from a minimum of 16 to a maximum of 24 experimental wells (in brackets), from 4 to 6 separate experimental sets. Results at statistical analysis (repeated-measures analysis of variance) are also shown.

*$P < 0.0001$ with respect to control; ethanol control; and 5-, 10, and 20-μmol/L α-TOS.

†$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, 20-, and 50-μmol/L α-TOS.

‡$P < 0.001$ with respect to control, ethanol control, and 5-μmol/L α-TOS.

§$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, 20-, 50-, and 100-μmol/L α-TOS.

‖$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, 20-, 50-, 100-, and 200-μmol/L α-TOS.

Cytotoxicity Experiments

Pancreatic cancer cell lines, C_2C_{12} myoblasts, monocytes, MEF-WT, MEF-DKO, and PSN1-SMAD4/DPC4+ cells were seeded (1000–2000 cells per well) in 96-well cell culture plates and cultured in complete media for 24 hours. Media were then replaced with fresh cell culture media with the addition of 1% or 10% FCS in the absence (control) or in the presence of increasing amounts (5, 10, 20, 50, 100, 200, and 500 μmol/L) of α-TOS (Sigma Chemical Co, St Louis, Mo), previously dissolved in 1% (vol/vol) ethanol; 24-, 48-, and 72-hour cell growth was assessed using the XTT cell viability test (Roche, Germany). All cell lines were also seeded (200–800 cells per well) in 96-well cell culture plates and cultured in complete media for 24 hours. Media were then replaced with fresh cell culture media with the addition of 10% FCS in the absence (control) or the presence of 0.0001-mmol/L 5-FU (Teva Pharma, Milano, Italy) alone or combined with 20- or 50-μmol/L α-TOS. Fresh media were replaced daily for 15 days. Cell growth was assessed every 2 days using the XTT cell viability test (Roche).

Statistical Analysis

The statistical analysis of data was made by means of repeated-measures analysis of variance using the SPSS statistical software for Windows version 9.0 (SPSS Inc, Chicago, Ill).

RESULTS

Effects of α-TOS on Pancreatic Cancer Cell Growth

The cell growth of BxPC3 (repeated-measures analysis of variance: F = 56.57; $P < 0.0001$), CAPAN1 (F = 127.7; $P < 0.0001$), MIA PaCa2 (F = 79.6; $P < 0.0001$), and PANC1 (F = 68.3; $P < 0.0001$) cultured in 1% FCS was significantly inhibited by 20-μmol/L α-TOS, whereas that of PSN1 was inhibited by lower α-TOS doses (10 and 5 μmol/L; F = 111.3; $P < 0.0001$). Higher α-TOS doses were required to reduce pancreatic cancer cell line growth, when cells were cultured in 10% FCS; the results obtained are reported in Table 1. In 10% FCS, the most α-TOS sensitive pancreatic cancer cell line, PSN1, was inhibited by 50 μmol/L, whereas a 100-μmol/L α-TOS was required for CAPAN1, MIA PaCa2, and PANC1.

α-Tocopheryl Succinate Effects on C_2C_{12} Myoblasts and Monocyte Cell Growth

C_2C_{12} myoblasts and normal monocytes obtained from blood donors were used to test the effects of α-TOS on nonneoplastic cells, which were cultured in the presence of 1% or 10% FCS, with or without increasing amounts of α-TOS. Cell growth was assessed using of the XTT test; the results obtained are reported in Table 2. The same α-TOS doses that inhibited pancreatic cancer cell growth inhibited also C_2C_{12} myoblasts cell growth:

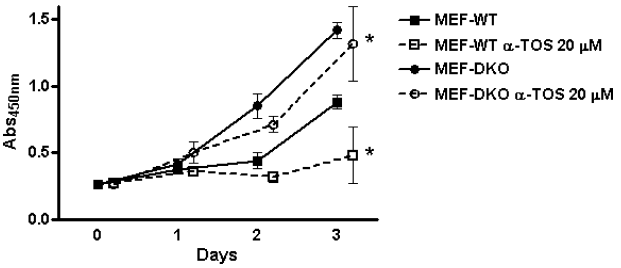

FIGURE 1. Sensitivity of WT and Bax/Bak DKO mouse embryonic fibroblast (MEF) cells to α-TOS. The MEF-WT (squares) and MEF-DKO (circles) cell growths in 1% FCS in the absence (continuous lines) and in the presence (dotted lines) of 20-μmol/L α-TOS. At days 1, 2, and 3, cell viability was analyzed by XTT assay ($Absorbance450_{nm}$). Results are from 6 separate experimental sets. *$P < 0.0001$ with respect to untreated cells.

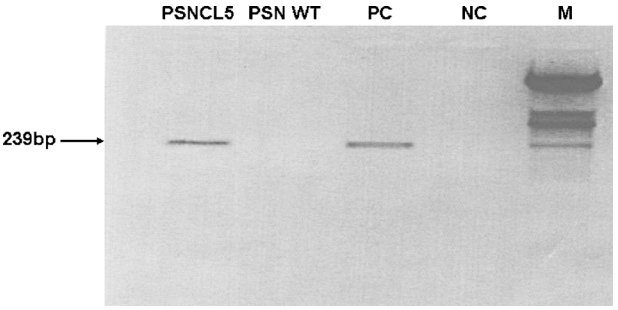

FIGURE 2. Reverse transcription PCR for *SMAD4/DPC4*. Expression of *SMAD4/DPC4* gene (239 base pairs) by RT-PCR analysis of PSN1 (PSN1 WT, line 2) and PSN1-*SMAD4/DPC4*+ cells after G418 selection (PSNCL5, line 1). Line 3, positive control (PC; MIA PaCa2); line 4, negative control (NC); and line 5, molecular weight marker (M).

50 μmol/L in 1% FCS and 100 μmol/L in 10% FCS. To inhibit monocyte cell growth, higher α-TOS doses were needed: 100 μmol/L in 1% FCS and 200 μmol/L in 10% FCS.

α-Tocopheryl Succinate Effects on MEF-WT and MEF-DKO Cell Growths

Both MEF-WT and MEF-DKO cells were cultured in the same experimental conditions as those described previously for pancreatic cancer, C_2C_{12} myoblasts, and monocytes. When MEF-WT cells were cultured in 1% FCS, their growth was completely inhibited by α-TOS of 50 μmol/L or more, whereas 20 μmol/L had a mild inhibitory effect (within-subject effect: F = 188.3; $P < 0.0001$ and between-subject effect: F = 409.5; $P < 0.0001$). In 10% FCS, a complete inhibition was obtained by 200 and 500 μmol/L, a reduced growth by 100-, 50-, and 20-μmol/L α-TOS (within-subject effect: F = 174.0; $P < 0.0001$ and between-subject effect: F = 413.5; $P < 0.0001$). Figure 1 shows MEF-WT

TABLE 3. Effects of α-TOS on Growth of PSN1 and PSN1-*SMAD4/DPC4*+ Cell Lines; Cells Were Cultured in RPMI With 10% FCS (Controls) With the Addition of Increasing Amounts of α-TOS

	Mean ± SD			
	Time 0	**24 h**	**48 h**	**72 h**
PSN1				
Control (n = 16)	0.34 ± 0.04	0.53 ± 0.08	1.04 ± 0.20	1.63 ± 0.29
Ethanol control (n = 16)	0.35 ± 0.04	0.52 ± 0.07	0.86 ± 0.18	1.38 ± 0.41
α-TOS				
5 μmol/L (n = 16)	0.34 ± 0.03	0.54 ± 0.06	0.95 ± 0.19	1.54 ± 0.23
10 μmol/L (n = 16)	0.34 ± 0.04	0.53 ± 0.08	0.87 ± 0.12	1.48 ± 0.23
20 μmol/L (n = 16)	0.36 ± 0.04	0.52 ± 0.06	0.85 ± 0.15	1.44 ± 0.26
50 μmol/L (n = 16)	0.35 ± 0.04	0.51 ± 0.07	0.72 ± 0.08	1.26 ± 0.24*
100 μmol/L (n = 16)	0.34 ± 0.02	0.44 ± 0.05	0.42 ± 0.02	0.40 ± 0.07†
200 μmol/L (n = 16)	0.35 ± 0.04	0.31 ± 0.03	0.25 ± 0.01	0.24 ± 0.01‡
500 μmol/L (n = 16)	0.36 ± 0.05	0.32 ± 0.04	0.29 ± 0.02	0.30 ± 0.02‡
Test for within-subject effect	F = 67.00; $P < 0.0001$			
Test for between-subject effect	F = 126.3; $P < 0.0001$			
PSN1-SMAD4/DPC4+				
Control (n = 24)	0.28 ± 0.01	0.48 ± 0.06	0.84 ± 0.08	1.72 ± 0.35
Ethanol control (n = 24)	0.30 ± 0.03	0.51 ± 0.05	0.85 ± 0.02	1.72 ± 0.26
α-TOS				
5 μmol/L (n = 24)	0.29 ± 0.01	0.47 ± 0.05	0.84 ± 0.05	1.75 ± 0.20
10 μmol/L (n = 24)	0.30 ± 0.03	0.45 ± 0.04	0.85 ± 0.06	1.61 ± 0.22
20 μmol/L (n = 24)	0.31 ± 0.05	0.47 ± 0.06	0.84 ± 0.09	1.70 ± 0.23
50 μmol/L (n = 24)	0.28 ± 0.01	0.45 ± 0.05	0.84 ± 0.03	1.65 ± 0.27
100 μmol/L (n = 24)	0.29 ± 0.01	0.46 ± 0.05	0.82 ± 0.02	0.90 ± 0.21†
200 μmol/L (n = 24)	0.30 ± 0.03	0.34 ± 0.03	0.48 ± 0.06	0.26 ± 0.01‡
500 μmol/L (n = 24)	0.31 ± 0.05	0.26 ± 0.03	0.24 ± 0.04	0.29 ± 0.02‡
Test for within-subjects effect	F = 146.6; $P < 0.0001$			
Test for between-subject effect	F = 340.9; $P < 0.0001$			

Because α-TOS was dissolved in 1% (vol/vol) ethanol, an ethanol control was always run in parallel. Cell growth was assessed by XTT assay before and 24, 48, and 72 hours after treatment. The results are reported as mean value ± SD of the $Absorbance450_{nm}$ obtained from a minimum of 16 to a maximum of 24 experimental wells (in brackets), from 4 to 6 separate experimental sets. The results at statistical analysis are also shown (repeated-measures analysis of variance).

*$P < 0.05$ with respect to control; ethanol control; and 5-, 10-, and 20-μmol/L α-TOS.

†$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, 20-, and 50-μmol/L α-TOS.

‡$P < 0.0001$ with respect to control; ethanol control; and 5-, 10-, and 20-, 50-, and 100-μmol/L α-TOS.

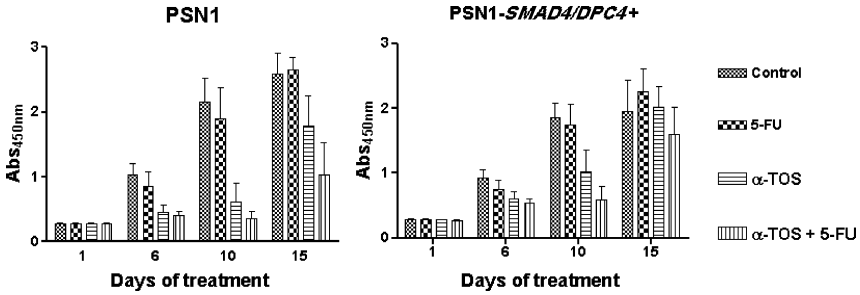

FIGURE 3. Sensitivity of PSN1 and PSN1-SMAD4/DPC4+ cell lines to the combined effects of α-TOS and 5-FU. PSN1 (left panel) and PSN1-*SMAD4/DPC4+* (right panel) cell lines were cultured for 15 days in RPMI with 10% FCS in the absence (control) or presence of nontoxic (0.0001 mmol/L) 5-FU alone or in combination with nontoxic (20 μmol/L) α-TOS. At days 1, 6, 10, and 15, cell viability was analyzed by XTT assay (Absorbance450_{nm}). Six separate experimental sets were performed. Columns represent mean, and bars represent SD.

and MEF-DKO growths when cultured in 1% FCS in the presence or absence of 20-μmol/L α-TOS, the lowest dose found to exert an inhibitory effect on MEF-WT cell growth. A similar pattern was observed when cells were cultured in 10% FCS (MEF-WT: within-subject effect: F = 34; $P < 0.0001$ and between-subject effect: F = 99.7; $P < 0.0001$; MEF-DKO: within-subject effect: F = 4.2; $P < 0.05$ and between-subject effect: F = 13.1; $P < 0.001$).

Effects of α-TOS on PSN1 and PSN1-*SMAD4/DPC4+* Cell Lines

Among the 5 pancreatic cancer cell lines studied, only BxPC3 had nonmutated *k-ras*, all had mutated *p53* and homozygous deleted *p16*, 2 had WT *DPC4/SMAD4* (MIA PaCa2 and PANC1), 1 had mutated *DPC4/SMAD4* (CAPAN1), and 2 (BxPC3 and PSN1) had homozygous *DPC4/SMAD4* deletion.[30,31] Of the 5 lines tested, PSN1 cells were the most sensitive to the toxic effects of α-TOS, and although they shared *DPC4/SMAD4* homozygous deletion with BxPC3, unlike the latter cell line, they also had mutated *k-ras*. The PSN1 cell line was selected to ascertain whether *DPC4/SMAD4* signaling protects cells against α-TOS toxicity. First, by reverse transcription polymerase chain reaction (RT-PCR) findings, we confirmed that SMAD4/DPC4 messenger RNA expression is absent in PSN1 cells (Fig. 2, line 2). After transfection and selection, *SMAD4/DPC4*-expressing clones (Fig. 2, line 1) were used for the subsequent series of experiments with α-TOS. The results obtained in PSN1 and PSN1-SMAD4/DPC4+ cells cultured in 10% FCS and treated or not treated (control) with increasing amounts of α-TOS are shown in Table 3. The expression of SMAD4/DPC4 reduced PSN1 cell sensitivity to α-TOS: the lowest dose able to inhibit PSN1 cell growth in 10% FCS was 50 μmol/L, whereas 100 μmol/L was required for PSN1-SMAD4/DPC4+ cells.

Effects of Combined α-TOS and 5-FU on Cell Lines

All the cell lines studied were cultured in 10% FCS for 15 days and remained untreated (control) or were treated with 0.0001-mmol/L 5-FU, a nonlethal dose previously established by us,[29] or with the same 5-FU dose combined with nontoxic (20 μmol/L) or mildly toxic (50 μmol/L) α-TOS doses. 5-Fluorouracil alone or combined with α-TOS did not significantly affect cellular growth in BxPC3, CAPAN1, MIA PaCa2, PANC1, C_2C_{12}, MEF-WT, and MEF-DKO cell lines, whereas combined treatment with nontoxic 5-FU (0.0001 mmol/L) doses and α-TOS (20 μmol/L) significantly reduced PSN1 cell growth (within-subject effect: F = 105.1; $P < 0.0001$ and between-subject effect: F = 658.2; $P < 0.0001$; Fig. 3, left panel). A higher α-TOS dose (50 μmol/L) per se inhibited the growth of this cell line. *SMAD4/DPC4* transfection partly restored PSN1 resistance to the effects of combined 5-FU and α-TOS effects on cell growth (within-subject effect: F = 57.5; $P < 0.0001$ and between-subject effect: F = 398.3; $P < 0.0001$; Fig. 3, right panel).

DISCUSSION

The redox-silent derivative of vitamin E, α-TOS, demonstrated to have antitumoral effects on several tumor types in vitro, including prostate, breast and gastric cancer, has been proposed as a potential new drug for cancer cure.[19–24] To investigate in vitro whether α-TOS antagonizes pancreatic cancer cell growth, we analyzed a series of 5 pancreatic cancer cell lines treated with a wide range of α-TOS doses, including the most commonly used 20-μmol/L concentration, reported to be safe for differentiated cells and toxic for cancer cells.[19,21,22] The growth of all 5 pancreatic cancer cell lines, cultured in both 1% and 10% FCS, was completely inhibited by the highest α-TOS doses used (200 and 500 μmol/L). Although this finding might seem promising, it is important to bear in mind that the high α-TOS doses blocking pancreatic cancer cell growth also had the same antiproliferative effect on nonneoplastic cells, monocytes, and C_2C_{12} myoblasts. Therefore, at the dose of 200 μmol/L or more, α-TOS is cytotoxic. If the compound is to be used in vivo, it is of vital importance to define doses that counteract pancreatic cancer cell growth but that do not compromise nonneoplastic cells. At 100 μmol/L, α-TOS had a mild inhibitory effect on the cell growth of 4/5 pancreatic cancer cell lines when cultured in 10% FCS, and it had no effect on these cells when administered at lower doses. This finding indicates that pancreatic cancer cells are more resistant to the antiproliferative effect of α-TOS than cells from other solid tumors: Neuzil et al[32] reported that at a dose of 50 μmol/L, the drug can significantly induce apoptosis in cancer of the lung and breast, bronchocarcinoma, and colon carcinoma cell lines cultured in 10% FCS; this finding was confirmed in the prostate cancer cell line LNCaP by Shiau et al[19] and in the gastric cancer cell line KATO-III by Rose et al.[21]

The findings made in the present study support the conclusions made by Ohlsson et al,[28] who found that primary pancreatic cancer cultures were insensitive to α-TOS. However, these authors tested α-TOS at a maximal concentration of 1 μmol/L, which was much lower than the concentrations used by us. In agreement with findings made in prostate cancer cell lines made by Shiau et al,[19] we found that the overall sensitivity of serum-deprived cells was greater than that of serum-stimulated cells: when these cells were cultured in 1% FCS, they became sensitive to an α-TOS concentration of 20 μmol/L, or less. This variation in sensitivity might be attributable to differences in the ability of cells to maintain mitochondrial integrity in response to the proapoptotic signals from α-TOS. Probably, as reported for prostate cancer cell lines, LNCaP and PC-3,[19] Bcl-x_L, expressed

by increasing the FCS concentration in culture media, protects cells from α-TOS-dependent apoptosis. The role of this anti-apoptotic member of the Bcl-2 family in regulating pancreatic cancer cell apoptosis has been well documented: (1) Bcl-x_L is expressed by untreated pancreatic cancer cell lines[33–35]; (2) when Bcl-x_L is silenced, pancreatic cancer cell sensitivity to the proapoptotic drug gemcitabine is increased[36]; and (3) Bcl-x_L overexpression is associated with the resistance that pancreatic cancer cells acquire toward proapoptotic chemotherapeutic agents.[36] The key role of the mitochondrial proapoptotic proteins, Bax and Bak, in mediating the antiproliferative effects of α-TOS, was confirmed in the present investigation on analyzing the response of MEF-WT and MEF-DKO cells. At the dose of 20 μmol/L, α-TOS inhibited MEF-WT cell growth when the cells were cultured in 1% FCS, and this effect was much less pronounced when Bax and Bak were knocked out (MEF-DKO).

The behavior of PSN1 was different from that of the other 4 pancreatic cancer cell lines, BxPC3, CAPAN1, MiaPaCa2, and PANC1: in the same experimental conditions (10% FCS), the growth of this cell line was markedly reduced after the 100-μmol/L α-TOS exposure and significantly delayed after the exposure to a dose of 50 μmol/L. The higher sensitivity to α-TOS of the PSN1 cell with respect to the other pancreatic cancer cell lines was confirmed by findings made on culturing the cells in growth factor deprived media (1% FCS): at a dose of 5 μmol/L, α-TOS inhibited PSN1 growth, whereas a higher dose (20 μmol/L) was required to achieve the same result in the 4 other cell lines. The higher sensitivity of PSN1 cells to the proapoptotic effect of α-TOS may be correlated with their genetic and phenotypic fingerprints, which differentiate them from the other pancreatic cancer cell lines. One featuring molecule might be density-enhanced phosphatase 1 (DEP-1)/HPTPn, a receptor-type protein tyrosine phosphatase, absent in BxPC3 and PANC1, and expressed at a barely detectable level in PSN1 and MIA PaCa2 cell lines.[37] The loss of this protein, associated with a more malignant phenotype, might cause resistance to proapoptogenic signals. A less relevant role might be played by high mobility group A1 (HMGA1), a protein involved in chromatin architecture and gene control and found to be highly expressed not only by PANC1 but also by the other pancreatic cancer cell lines, including PSN1.[38] On considering oncogenes and tumor suppressor gene mutations in the same 5 pancreatic cancer lines as those investigated in the present article, Moore et al[30] reported that PSN1 was the only line to bear all the following: *k-ras* mutation, p53 mutation, and homozygous deletion of *SMAD4/DPC4*. Our findings at RT-PCR analysis confirm that PSN1 had *SMAD4/DPC4* homozygous deletion. To ascertain the role of *SMAD4/DPC4* in mediating sensitivity to α-TOS, SMAD4/DPC4-expressing PSN1 clones obtained after chemical transfection were treated as parental PSN1 cells: the restoration of *SMAD4/DPC4* expression was accomplished by a reduced sensitivity to α-TOS effects. This indicates that the antiproliferative effects of α-TOS depend not only on mitochondrial integrity but also on the loss of *SMAD4/DPC4*. However, although TGF-β/SMAD signaling plays a central role in tumorigenesis of several epithelia, it switches paradoxically from suppressing tumor cells to promoting metastatic cells during cancer progression.[39] It was recently demonstrated that an intact TGF-β/SMAD signaling plays a central role in concert with mutant p53 in counteracting the protective effects of p63 against apoptosis.[14] SMAD4/DPC4 deletion might therefore increase tumor cell sensitivity to proapoptotic signals.

The findings made in the present study show that, overall, the effect of α-TOS was minimal in pancreatic cancer cells. We tested whether this compound sensitizes pancreatic cancer cells to the chemotherapeutic agent 5-FU, by treating pancreatic cancer cells with nontoxic doses of both compounds. We found that all 4 cell lines with a low sensitivity to α-TOS were insensitive to the combined action of α-TOS and 5-FU, whereas the growth of the α-TOS–sensitive cell line, PSN1, was abolished in long-term experiments (15-day follow-up), and it was confirmed that this effect depends upon *SMAD4/DPC4* deletion. Therefore, the exposure to a safe α-TOS dose (20 μmol/L in 10% FCS), unable per se to reduce PSN1 cell growth, confers them an extreme sensitivity to nontoxic doses (0.0001 mmol/L) of 5-FU. This result has important potential clinical implications: the analysis of *SMAD4/DPC4* expression might allow the identification of tumors for which combined α-TOS and 5-FU would be effective at well-tolerated doses.

In conclusion, only a minority of pancreatic cancer cells are sensitive to the antiproliferative effects of α-TOS, any sensitivity appearing to be correlated with *SMAD4/DPC4* homozygous deletion. If applied to clinical practice, the finding that nontoxic α-TOS doses cause tumor cells with *SMAD4/DPC4* deletion to become sensitive to 5-FU may offer a chance of effective chemotherapy to patients with pancreatic cancer.

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