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ORIGINAL ARTICLE

Enhancing cytotoxic agent activity in experimental pancreatic cancer through EMAP II combination therapy

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Abstract
Purpose Gemcitabine has limited benefits as single agent or in combination for pancreatic ductal adenocarcinoma (PDAC). Endothelial monocyte-activating polypeptide II (EMAP) enhances gemcitabine effects in PDAC. We evaluated the combination effects of EMAP, doxorubicin, and docetaxel in experimental PDAC.
Methods Cell proliferation, protein expression, and apoptosis were analyzed by WST-1 assay, Western blotting, and FACS analysis. Tumor growth and survival experiments were performed in murine xenografts.
Results PDAC cell proliferation in vitro was not affected by EMAP, compared to a small inhibition through doxorubicin, docetaxel, and gemcitabine. EMAP addition to these agents did not increase the antiproliferative effects. In endothelial cells, EMAP, doxorubicin, docetaxel, and gemcitabine all had antiproliferative effects. Addition of EMAP to these cytotoxic agents had additive effects. In PDAC cells, no agent induced measurable apoptosis, whereas in endothelial cells, all agents either alone or in combination did. Doxorubicin, docetaxel, gemcitabine, and EMAP all decreased tumor growth. EMAP addition increased inhibitory effects of docetaxel and gemcitabine, but not of doxorubicin. However, compared to controls (median survival: 17 days), EMAP (14 days) had no survival benefit, while docetaxel (29 days), gemcitabine (25 days), and docetaxel followed by gemcitabine sequence (37 days) extended animal survival. Addition of EMAP to docetaxel (35 days), gemcitabine (28 days), and docetaxel gemcitabine sequence (41 days) extended the survival. Doxorubicin effects were not enhanced by EMAP.
Conclusions The antiendothelial combination therapy benefit through EMAP is not limited to gemcitabine and may facilitate the development of more effective alternative cytotoxic therapy strategies against PDAC.

Keywords Pancreatic cancer · EMAP II · Doxorubicin · Docetaxel · Gemcitabine · Combination therapy

Abbreviations

PDAC	Pancreatic ductal adenocarcinoma
EMAP or E	Endothelial monocyte-activating polypeptide II
Gem	Gemcitabine
Dox	Doxorubicin
DT	Docetaxel
PARP-1	Poly (ADP-ribose) polymerase-1
WST-1	(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate)
EC	Endothelial cell

Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains among the leading causes of cancer-related deaths in the United

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States [14]. Among the reasons for the high lethality of PDAC are the limited ability for an early diagnosis, the aggressive nature of the tumor, and its high resistance to most systemic therapies. Approximately 80% of patients have advanced-stage local or distant metastatic disease at the time of diagnosis. The median survival is 6–10 months for patients with locally advanced disease and 3–6 months for patients with metastatic disease [25]. Currently, single-agent gemcitabine (Gem) is the standard treatment of advanced PDAC based on clinical benefits and prolonged survival of patients when compared with 5-FU [8]. However, an objective gemcitabine treatment response is only observed in the minority of PDAC patients.

There are an increasing number of studies focusing on combining gemcitabine with other cytotoxic or targeted-therapy agents. However, most of these approaches failed to show any significant improvement in objective tumor response compared to single-agent gemcitabine [4–6, 11, 13, 18, 20, 21, 24]. A combination of gemcitabine and the epidermal growth factor receptor inhibitor erlotinib has shown some very modest improvement in survival over gemcitabine alone; however, erlotinib addition was associated with a higher incidence of rash, diarrhea, and hematologic toxicity [22].

Doxorubicin (Dox), a quinone-containing anthracycline antibiotic, is a highly potent antitumor agent. It binds to DNA by intercalation and induces a DNA damage response. Doxorubicin is used to treat hematologic and solid cancers such as breast and ovarian cancers and many other solid tumors, although its role for PDAC therapy is limited [17]. Docetaxel (DT) is an antimitotic agent that interferes with the dynamics of microtubule assembly leading to cell-cycle arrest and apoptosis. It is a semi-synthetic taxane with a broad spectrum of antitumor activity, with uses against breast, ovarian, and non-small cell lung cancers. Docetaxel has been reported to have modest single-agent activity in PDAC [19].

Tumor progression requires complex interactions among tumor cells, extracellular matrix components, endothelial cells (ECs), and stromal cellular components including inflammatory cells and fibroblasts. Targeting ECs for solid tumor treatment has demonstrated substantial benefit in an experimental model [12]. The mature form of endothelial monocyte-activating polypeptide II (EMAP II, EMAP) is a proinflammatory cytokine with antiangiogenic activities. EMAP has potent effects of ECs, as it has been shown to induce apoptosis in ECs and to inhibit proliferation, vascularization, and neovessel formation [2, 27]. EMAP has also been found to suppress primary and metastatic tumor growth, in part via its antiangiogenic properties [27, 31, 34]. The in vivo antitumor effects of EMAP may be explained by its ability to bind to $\alpha5\beta1$ integrin and VEGF receptors, leading to an interference in integrin–fibronectin (FN) interactions and in VEGF signaling [1, 29]. Our recent studies have shown that EMAP improves gemcitabine effects against experimental PDAC, with further improvement in antitumor effects when combined with gemcitabine and the anti-VEGF agent bevacizumab [30, 32]. Based on these findings, we extended the combination testing with EMAP in PDAC treatment to the other chemotherapeutic agents doxorubicin and docetaxel and compared these with gemcitabine to identify whether EMAP combination benefits are agent-specific and whether the activity of other agents could be enhanced to support potential PDAC clinical applications.

Materials and methods

Materials

Recombinant human EMAP was prepared and purified as previously described [28]. Gemcitabine was purchased from Eli Lilly Corporation (Indianapolis, IN). Doxorubicin (Doxil®) was purchased from Ben Venue Laboratories, Bedford, OH, and Docetaxel (Taxotere®) was purchased from Sanofi Aventis, Bridgewater, NJ. The cell proliferation reagent WST-1 was purchased from Roche Diagnostic Corporation (Indianapolis, IN).

Cell culture

The human PDAC cell line AsPC-1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 medium (Sigma Chemical Co. St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. Primary human umbilical vein endothelial cells (HUVECs) were grown in E-stim medium, containing EC growth supplement and epidermal growth factor (BD Biosciences, Bedford, MA), at 37°C in humidified 5% CO_2 atmosphere.

Cell viability assay

Cell viability of AsPC-1 and HUVECs was evaluated by the colorimetric WST-1 assay. The measurement is based on the ability of viable cells to cleave the sulfonated tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases. AsPC-1 and HUVECs (4,000 cells per well) were plated in a 96-well plate in regular growth medium, and after 16 h, the medium was replaced with 2% FBS containing RPMI 1640 for AsPC-1 cells or EBM-2 basal EC medium for HUVECs, respectively. After 5-h incubation, the cells were treated with doxorubicin, docetaxel, and gemcitabine, with or without EMAP. The range of concentrations used (10 nM–10 μM) for all agents was comparable to clinically

achievable concentration. After additional incubation of 72 h, 10 μl WST-1 reagent was added in each well followed by incubation for 2 h. The absorbance at 450 nm was measured using a microplate reader.

Western blot analysis

A monolayer of cells at 75–80% confluence was placed in 2% FBS containing medium for AsPC-1 cells and in EBM-2 medium for HUVECs for at least 5 h before treatment with doxorubicin (10 μM), docetaxel (10 μM), gemcitabine (10 μM), or EMAP (10 μM) for 24 h. Cells were lysed, and equal amounts of total protein were separated by SDS–PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked for 1 h at room temperature in TBS-T (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20). The membranes were incubated overnight at 4°C with the following antibodies: phospho-JNK (Santa Cruz Biotechnologies, Santa Cruz, CA), poly (ADP-ribose) polymerase-1 (PARP-1) (Cell Signaling Technology, Beverly, MA) or GAPDH (Sigma). The membranes were then incubated with corresponding HRP-conjugated secondary antibodies (Pierce Biotechnologies, Santa Cruz, CA) for 1 h at room temperature. Specific bands were detected using the enhanced chemiluminescence reagent (ECL, Perkin Elmer Life Sciences, Boston, MA) on autoradiographic film and quantitated by densitometry.

Apoptosis assay by annexin V-FITC and propidium iodide (PI) staining

Effects on in vitro cell apoptosis were detected by using the annexin V-FITC apoptosis detection kit (BioVision, CA) as per manufacturer's protocol. Briefly, AsPC-1 cells or HUVECs were grown up to 80% confluence, medium was changed to low serum-containing medium and incubated for 5 h and then treated with doxorubicin (10 μM), docetaxel (10 μM), gemcitabine (10 μM), or EMAP (10 μM) for 12 h. After treatment incubation, cells were trypsinized and suspended in 1 ml of low serum medium. From each cell suspension, 2×10^5 cell were centrifuged and resuspended in 500 μl 1X binding buffer. In each sample, 5 μl of annexin V-FITC and 5 μl of PI was added, incubated for 5 min in dark, and immediately analyzed by flow cytometry (FACS Calibur System, BD Biosciences, Franklin Lakes, NJ). The annexin-V-FITC positive and PI-negative cells were enumerated as early apoptotic cells.

In vivo tumor growth experiment

All animal studies were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center (Dallas, TX). Athymic female nude mice were used to establish the subcutaneous xenograft model for in vivo tumor growth. AsPC-1 cells were suspended in serum-free RPMI 1640 medium, and 0.75×10^6 cells were subcutaneously injected per mouse. After 14 days, all mice had measurable tumor. The animals were randomly grouped ($n = 6$–8 per group) and treated intraperitoneally with PBS (control), EMAP (80 μg/kg in 100 μl PBS daily), doxorubicin (3 mg/kg in 100 μl PBS, twice weekly), docetaxel (3 mg/kg in 100 μl PBS, twice weekly), and gemcitabine (100 mg/kg in 100 μl PBS, twice weekly), either alone or in combination for 14 days. The tumor size of all mice was measured twice weekly by caliper. The tumor volume was estimated using the formula [$V = \pi h(h^2 + 3 \infty^2)/6$], where V = volume, h = height, and ∞ = (length + width)/2. Net growth in tumor size was calculated by subtracting tumor volume on the first day of treatment from that on the last day. After completion of treatment, all animals were euthanized, and tumors were removed, weighed, dissected, and processed for histological or immunohistochemical analysis.

Histology and immunohistochemical analysis

Tumor tissue specimens fixed in 4% paraformaldehyde and embedded in paraffin were used for histological and immunohistological analysis. Intratumoral proliferative activity was measured using by Ki67 nuclear antigen staining as per manufacturer's protocol (Abcam, Cambridge, MA). Briefly, paraffin-embedded tumor tissue sections were cut, deparaffinized, rehydrated, and antigen-retrieved with citrate buffer. The tissue sections were incubated with CAS blocking buffer for 20 min followed by 1-h incubation with 100 μl Ki67 antibody (1:200 dilution in CAS buffer). After PBS wash, the tissue sections were then incubated with 200 μl Cy3 (1:200 dilution) secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 40 min at room temperature. After PBS wash, slides were mounted using mounting solution containing DAPI (Invitrogen, Carlsbad, CA). Intratumoral proliferative activity was evaluated by calculating Ki67-stained cells from five different high-power fields (HPF) in a blinded manner.

Animal survival analysis

In vivo animal survival studies were performed using 4- to 6-week-old SCID mice purchased from an on-campus facility. Animal survival experiments were carried out as previously reported [33]. Briefly, female SCID mice were injected intraperitoneally with 0.75×10^6 AsPC-1 cells in 100 μl serum-free RPMI 1640 medium. Two weeks after tumor cell injection, animals were randomly grouped ($n = 6$–8 per group) and treated intraperitoneally with PBS

(control), EMAP (80 μg/kg in 100 μl PBS daily), doxorubicin (3 mg/kg in 100 μl PBS, twice weekly), docetaxel (3 mg/kg kg in 100 μl PBS, twice weekly), and gemcitabine (100 mg/kg in 100 μl PBS, twice weekly), either alone or in combination, for 2 or 3 weeks. Animals were euthanized when turning moribund. Survival was evaluated from the first day of treatment until death.

Statistical analysis

Results from in vitro cell proliferation assays are expressed as mean ± standard deviation (s.d.), and group data were compared by two-tailed Student's *t*-test. For all in vivo measurements, statistical analyses were performed using ANOVA for multiple group comparisons and Student's *t*-test for individual group comparisons. Statistical differences in survival studies were analyzed by non-parametric survival statistics and Peto-Wilcoxon testing. Group differences were considered statistically significant at a *P* value of <0.05.

Results

Effects of doxorubicin, docetaxel, gemcitabine, and EMAP on in vitro cell proliferation

Analysis of in vitro AsPC-1 cell proliferation revealed that single agent EMAP had no effect, while doxorubicin and gemcitabine caused modest ($IC_{50} > 10$ μM) and docetaxel caused major inhibitory effects ($IC_{50} = 8$ μM) on cell proliferation. At 10 μM concentration, the percentage of inhibition in AsPC-1 cell proliferation by EMAP, doxorubicin, docetaxel, and gemcitabine treatment was 1.4, 26.5, 60, and 40, respectively. Addition of EMAP (10 μM) to different concentrations of doxorubicin, docetaxel, or gemcitabine did not increase the inhibitory effect of either agent on cell proliferation (Fig. 1a). In HUVECs, all agents including EMAP caused significant reduction in cell proliferation. At 10 μM concentration, the percentage of inhibition in HUVEC proliferation by EMAP, doxorubicin, docetaxel, and gemcitabine was 59, 79, 96, and 85, respectively. Significant additive antiproliferative effects were observed by the addition of EMAP (10 μM) to all concentrations of doxorubicin, docetaxel, and gemcitabine (Fig. 1b).

Effect of doxorubicin, docetaxel, gemcitabine, and EMAP on apoptosis

Detection of early apoptotic cells measured by annexin/PI staining revealed that in the PDAC cell line AsPC-1, doxorubicin, docetaxel, gemcitabine and EMAP did not cause measurable induction in apoptosis at a 10 μM concentration. The percentage of early apoptotic cells in doxorubicin-, docetaxel-, gemcitabine-, and EMAP-treated groups was 6, 5.3, 5, and 4.5, respectively. Addition of EMAP to doxorubicin, docetaxel, or gemcitabine groups did not cause any significant induction in apoptosis in AsPC-1 cells; the percentage of early apoptotic cells in Dox + E, DT + E, and Gem + E combinations was 7.8, 5.3, and 6.2, respectively (Fig. 2a). In contrast, all agents caused an increase in early apoptotic cells in HUVECs, as at a 10 μM concentration, the percentage of early apoptotic cells in doxorubicin-, docetaxel-, gemcitabine- and EMAP-treated groups was 24.2, 19.1, 19.8, and 20, respectively. Addition of EMAP to doxorubicin, docetaxel, and gemcitabine group had only small additive effects, leading to a percentage of early apoptotic cells in Dox + E, DT + E, and Gem + E groups of 26.3, 20.2, and 20.4, respectively (Fig. 2b).

Effects of doxorubicin, docetaxel, gemcitabine, and EMAP on apoptosis-related proteins

Regulation of apoptosis-related proteins in form of PARP-1 cleavage and phospho-JNK expression was investigated after doxorubicin, docetaxel, gemcitabine, and EMAP treatment. Western blot analysis of AsPC-1 cell lysates revealed that doxorubicin, docetaxel, or gemcitabine caused no significant increase in either PARP-1 cleavage or phospho-JNK expression (Fig. 3a). In HUVECs, doxorubicin, docetaxel, gemcitabine, and EMAP caused a 5.2-, 2.2-, 2.2-, and 2.3-fold increase in PARP-1 cleavage compared to controls, respectively. EMAP mediated additive effects in the doxorubicin and docetaxel groups but not to gemcitabine; increase in PARP-1 cleavage in the Dox + E, DT + E, and Gem + E groups compared to controls was 6.1-, 3-, and 1.5-fold, respectively (Fig. 3b). Phospho-JNK expression was increased by EMAP and doxorubicin, but not by docetaxel or gemcitabine. In all EMAP-containing combinations, the phospho-JNK expression was greater than in the corresponding single-agent settings (Fig. 3b).

Effects of doxorubicin, docetaxel, gemcitabine, and EMAP on heterotopic localized tumor growth

In the human AsPC-1 PDAC cell xenograft model, doxorubicin, docetaxel, gemcitabine, and EMAP all decreased local tumor growth as single agents. Addition of EMAP increased the inhibitory effects of docetaxel and gemcitabine but not of doxorubicin. Sequential treatment with docetaxel followed by gemcitabine led to a maximum inhibition in tumor growth, which again was further increased by EMAP addition (Fig. 4a). Analysis of net growth over 21 days revealed that the relative inhibition compared to controls after treatment with doxorubicin,

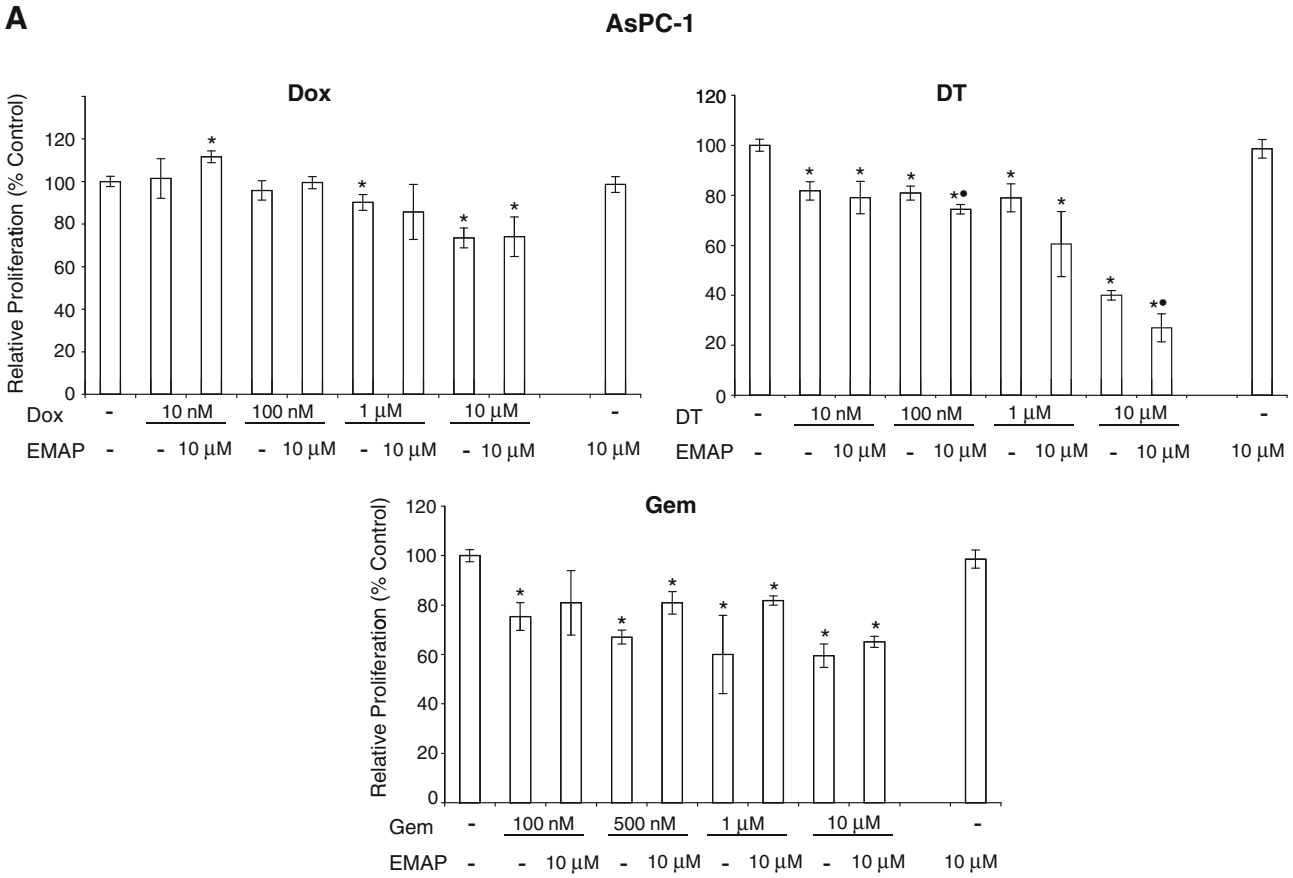

Fig. 1 Effect of doxorubicin (Dox), docetaxel (DT), gemcitabine (Gem), and EMAP II (EMAP) on in vitro cell proliferation. AsPC-1 cells (**a**) and HUVECs (**b**) were plated on 96-well plates and were treated with PBS (control), doxorubicin, or docetaxel (10 nM, 100 nM, 1 μM, or 10 μM) or gemcitabine (100 nM, 500 nM, 1 μM, or 10 μM), with or without fixed dose of EMAP (10 μM). After 72 h, 10 μl WST-1 reagent was added in each well and incubated for 2 additional hours. The resulting number of viable cells was calculated by measuring absorbance of color produced in each well. Data are representative of mean values ± SD of quadruplicate determinants. Symbol * represents significant differences ($P < 0.05$) of all treatment groups compared to control. Symbol • represents significant differences ($P < 0.05$) of Dox, DT, or Gem treatment groups in combination with EMAP compared to their respective concentrations without EMAP

docetaxel, gemcitabine, or EMAP was approximately 92, 63, 60, and 42 percent, respectively (corresponding *P* values: 0.002, 0.04, 0.05, and 0.17). EMAP exerted additive effects on docetaxel and gemcitabine but not on doxorubicin; compared to net tumor growth in controls, that after Dox + E, DT + E and Gem + E was 73, 85, and 68 percent, with *P* values of 0.002, 0.003, and 0.004, respectively (Fig. 4b). In addition, compared to controls, net tumor growth inhibition in the sequential treatment with docetaxel followed by gemcitabine was 72% without EMAP, but 99% with EMAP, with *P* values of 0.009 and 0.001, respectively (Fig. 4b). Differences in tumor growth inhibition with and without EMAP after treatment with doxorubicin, docetaxel, gemcitabine, or docetaxel followed by gemcitabine did not reach statistical significance because of the limited animal number in each group. No apparent signs of drug toxicity were observed in any treatment group in terms of animal habitus, weight, and activity levels.

Effects of doxorubicin, docetaxel, gemcitabine, and EMAP on intratumoral proliferative activity

In the analysis of intratumoral proliferative activity within tumor tissue sections, the groups treated with EMAP, doxorubicin, docetaxel, gemcitabine, and docetaxel followed by gemcitabine all displayed a significant reduction in ki67-positive cells. Addition of EMAP had additive effects on reducing ki67-positive proliferative cells in animals treated with docetaxel, gemcitabine, or docetaxel followed by gemcitabine, but not after doxorubicin (Fig. 5a). Quantitation of proliferative activity performed by counting ki67 positive cells versus total cells per high-power field revealed that groups treated with EMAP, doxorubicin, docetaxel, gemcitabine, or docetaxel followed by gemcitabine showed a more than 39, 51, 50, 48, and 57 percent reduction in proliferative activity, respectively (*P* values: 0.015, 0.005, 0.002, 0.005, and 0.003). Addition of EMAP enhanced the

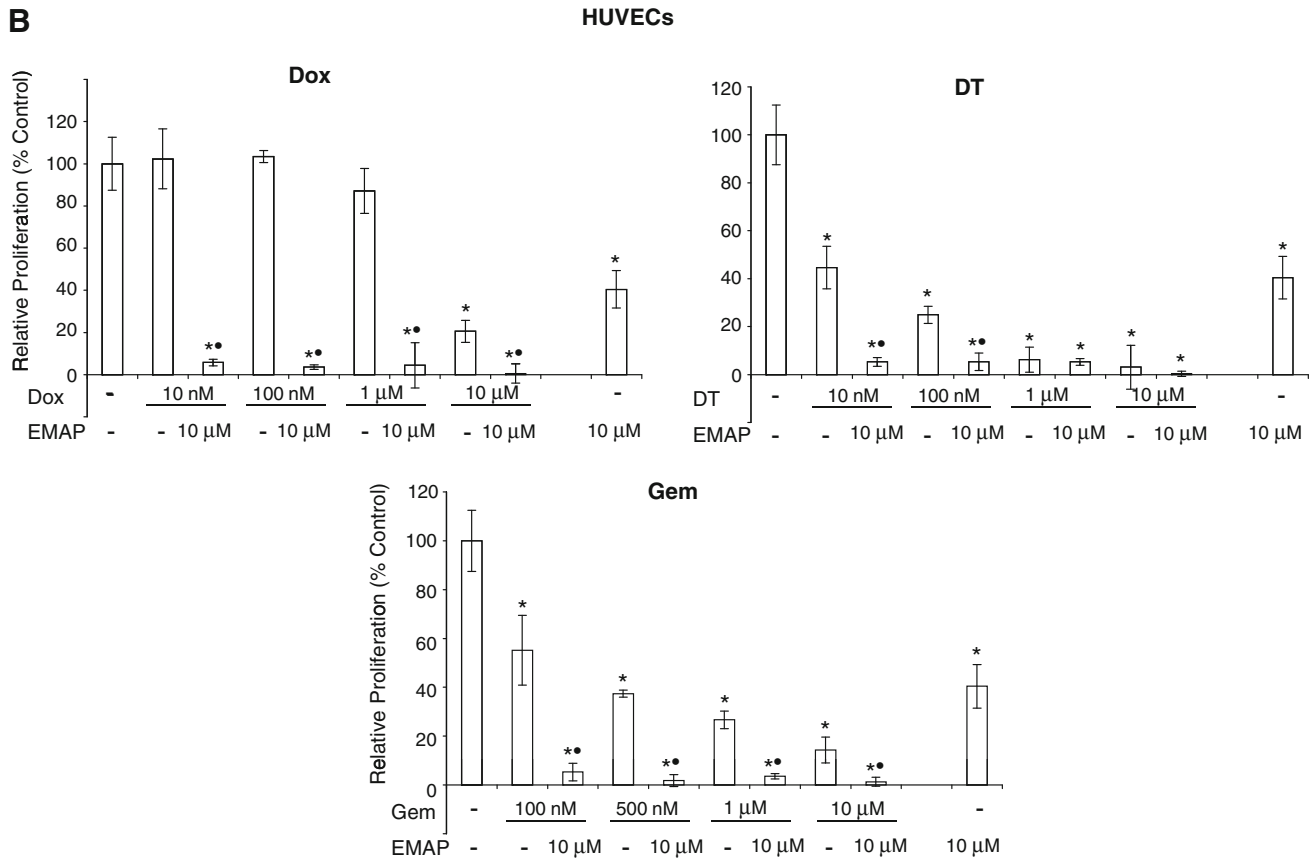

Fig. 1 continued

effects of docetaxel, gemcitabine, and docetaxel followed by gemcitabine, but not after doxorubicin; compared to controls, proliferative activity in Dox + E, DT + E, Gem + E, and DT followed by Gem + E group was 49, 66, 66, and 89 percent with *P* values of 0.004, 0.0004, 0.002, and 0.00005, respectively (Fig. 5b). The *P* values for statistical comparisons of single agents with EMAP in addition to doxorubicin, docetaxel, gemcitabine, and docetaxel followed by gemcitabine were 0.87, 0.007, 0.09, and 0.0001, respectively.

Effects of doxorubicin, docetaxel, gemcitabine, and EMAP on animal survival

Animal survival studies performed with human AsPC-1 PDAC xenografts in SCID-NOD mice resulted in a median survival of 21 days in controls and of 20 days after EMAP (P = n.s.); doxorubicin (31 days, $P = 0.01$) and docetaxel (35 days, $P = 0.003$) monotherapy extended the resulting survival. EMAP enhanced the docetaxel survival effect in combination (median: 44 days, $P = 0.009$ vs. controls), but not of that of doxorubicin (median: 31 days, $P = 0.04$ vs. controls) (Fig. 6a).

In a separate experiment, survival effects of gemcitabine and docetaxel were measured as monotherapy and as sequential therapy, both with and without EMAP. Compared to controls, gemcitabine, docetaxel, gemcitabine followed by docetaxel, and docetaxel followed by gemcitabine, all increased animal survival; the median survival was 25, 29, 39, and 39 days, respectively, in comparison with 17 days in controls (*P* values: 0.02, 0.01, 0.003, and 0.001). Addition of EMAP extended the survival benefit of gemcitabine, docetaxel, and docetaxel followed by gemcitabine, but not in the group treated with gemcitabine followed by docetaxel. The median survival results after Gem + E, DT + E, sequential Gem/DT + E, and sequential DT/Gem + E were 28 (*P* vs. controls = 0.003), 35 ($P = 0.02$), 34 ($P = 0.005$), and 41 days ($P = 0.001$), respectively (Fig. 6b). Comparisons of corresponding groups without and with EMAP failed to reach significance levels. As in the previous experiments, there was no sign of drug-related toxicity.

Discussion

Pancreatic cancer is clinically characterized by early metastatic activity, late-stage diagnosis, and a general resistance to cytotoxic chemotherapy. Gemcitabine is currently considered the standard chemotherapeutic agent for PDAC,

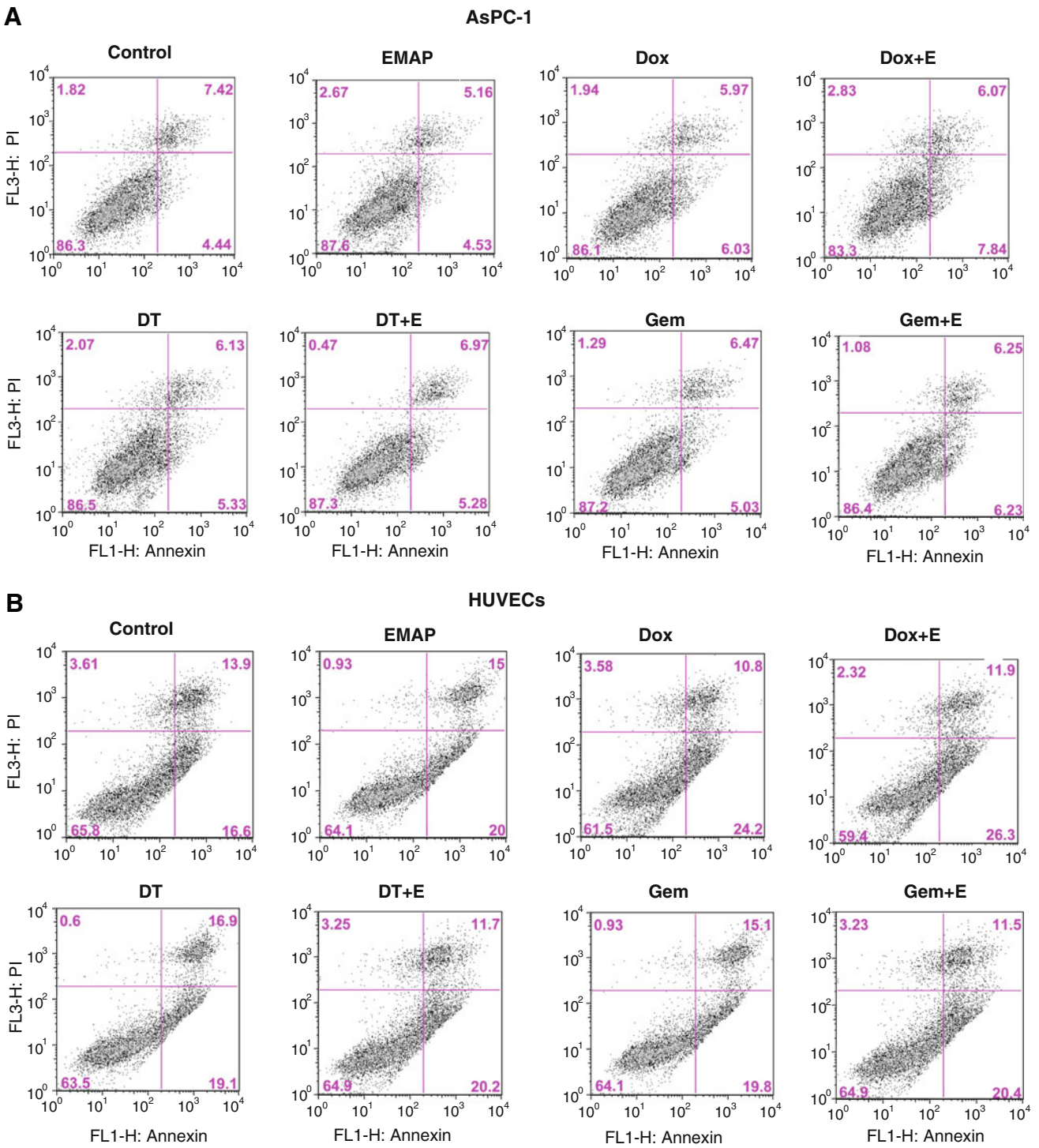

Fig. 2 Effects of doxorubicin (Dox), docetaxel (DT), gemcitabine (Gem), and EMAP (E) on pancreatic cancer and endothelial cell apoptosis. AsPC-1 cells (**a**) and HUVECs (**b**) were treated with Dox (10 μM), DT (10 μM), and Gem (10 μM), either alone or in combination with E (10 μM). The early apoptotic cells (lower right quadrant) were measured by flow cytometry after staining with FITC-conjugated annexin V and propidium iodide. The percentage of cells in each quadrant is indicated within the quadrant. Data are representative of two independent experiments with similar results

but gemcitabine treatment either alone or in combination with other chemotherapeutic drugs only imparts limited benefits in terms of overall survival [5, 20, 24]. Combinations of gemcitabine with antiangiogenic agents also failed to improve survival of pancreatic cancer patients [7, 10, 13, 15]; only the gemcitabine plus erlotinib (EGF

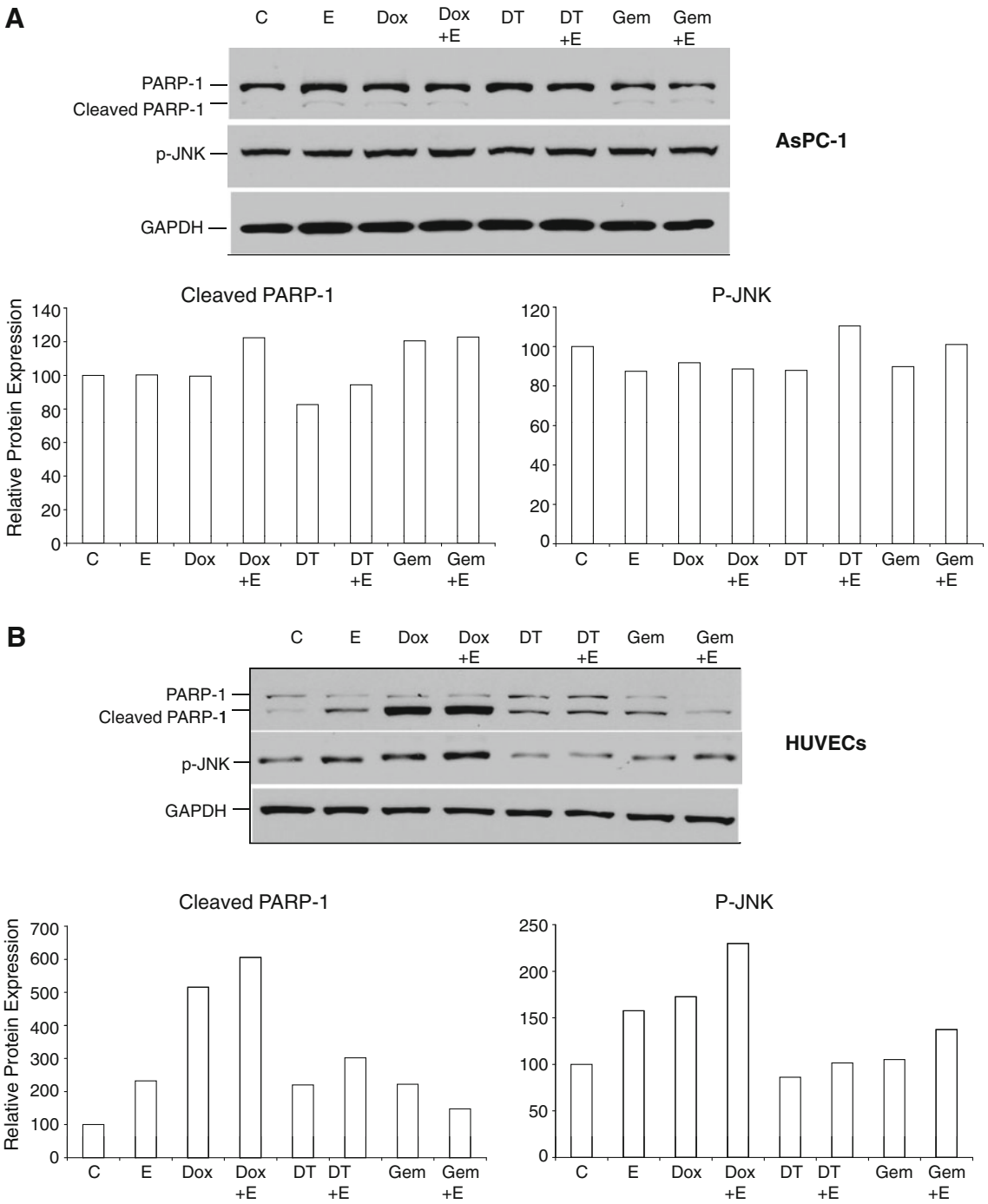

Fig. 3 Effects of doxorubicin (Dox), docetaxel (DT), gemcitabine (Gem), and EMAP (E) treatment on PARP-1 cleavage and phospho-JNK expression. A monolayer of sub-confluent AsPC-1 cells (**a**) and HUVECs (**b**) were treated for 24 h with Dox (10 μM), DT (10 μM), and Gem (10 μM), either alone or in combination with EMAP (10 μM). Western blot bands represent the cellular expression of the full-length PARP-1 (116 kDa), cleaved PARP-1 (89 kDa), phospho-JNK (55 kDa), and control housekeeping protein GAPDH (37 kDa). The intensity of bands was quantitated by densitometry and is represented in the bar graph after normalizing values with GAPDH expression. Data are representative of two independent experiments with identical conditions

receptor inhibitor) combination showed a small survival benefit [22]. The high resistance of PDAC to all current therapeutic strategies is likely multifactorial due to the complexity of intratumoral pathway alterations that support tumor progression in parallel. Therefore, one possible approach to improve PDAC treatment is the development

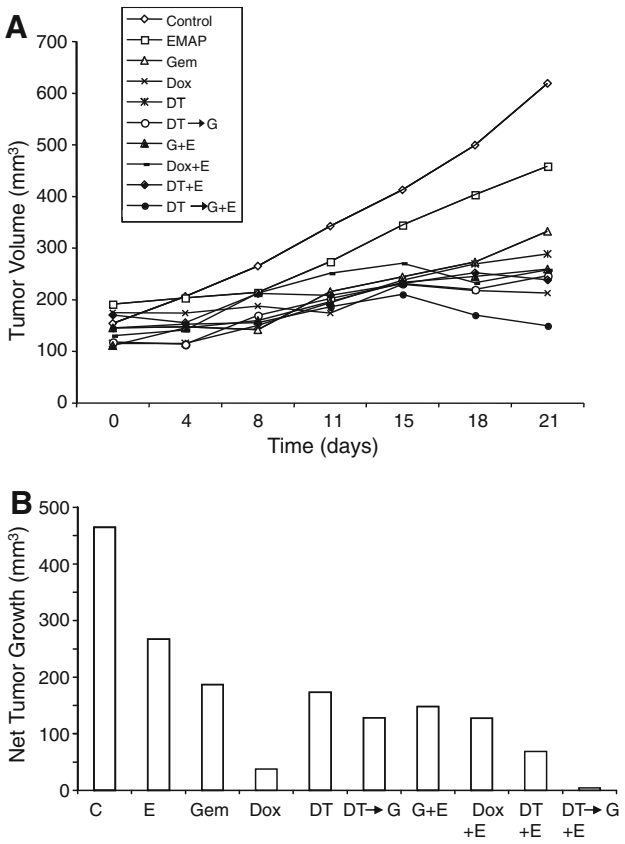

Fig. 4 Effects of doxorubicin (Dox), docetaxel (DT), gemcitabine (Gem), and EMAP (E) treatment on local tumor growth. AsPC-1 cells (0.75×10^6) were injected subcutaneously into nude mice. Two weeks after tumor cell injection, mice were treated with PBS (control), Dox, DT, Gem, and E, either alone or in combination, for 3 weeks. Sequential treatment with DT first followed by Gem has been labeled as DT → G. **a** Tumor size was measured twice weekly with calipers. **b** Net tumor growth was calculated by subtracting tumor volume on the first day of treatment from that on the last day. Data are representative of mean values ± SD ($n = 6$–8 mice per group)

of combination therapies that target specific molecular components to block more than one of the multiple mechanisms of PDAC progression.

As seen in many other tumor types, angiogenesis plays a critical role in the progression of primary and metastatic PDAC. Currently, several antiangiogenic strategies are being explored for their direct antitumor potential, toxicity, and synergistic interaction with other antitumor agents [16, 26]. Tumor angiogenesis critically depends on EC recruitment, adhesion, survival, proliferation, and migration. Targeting ECs for treatment of solid tumors has been demonstrated to be of substantial benefit [12]. EMAP, a tumor-derived cytokine, has antiangiogenic, anti-endothelial, and antitumor effects in several tumor types including PDAC [9, 23, 27, 31]. Antitumor effects of EMAP can be attributed to its range of activities including the inhibition of FN- and VEGF-induced angiogenesis signaling [1, 31], antiproliferative, and pro-apoptotic effects on ECs [27, 36] and a decrease in intratumoral VEGF expression [34]. EMAP has also enhanced the antitumor activities of gemcitabine [32] or gemcitabine in combination with bevacizumab [30]. We therefore explored any possible combination benefits of EMAP with other chemotherapeutic agents such as doxorubicin and docetaxel regarding experimental pancreatic cancer survival. In the study presented here, we observed a small in vitro effect of doxorubicin, docetaxel, and gemcitabine on pancreatic cancer AsPC-1 cell proliferation or apoptosis, upon which EMAP addition had no effect. Conversely, all these agents inhibited cell proliferation and induced apoptosis in endothelial cells, with EMAP exhibiting additive effects on these activities. It appears thus likely that in vivo antitumor effects of EMAP are at least in part due to its effects on ECs and perhaps other stromal components, but not primarily directed against the PDACs. Our in vivo experiments showed that all cytotoxic agents and EMAP inhibited tumor growth alone; EMAP addition to these agents increased the antitumor effects of docetaxel and gemcitabine but not of doxorubicin, and even the maximum cytotoxic effect achieved after sequential treatment with docetaxel followed by gemcitabine was further enhanced by EMAP addition. These findings were further corroborated by the analysis of intratumoral proliferative activity. Interestingly, EMAP alone reduced the intratumoral proliferative activity and enhanced the antiproliferative docetaxel and gemcitabine effects, a finding that is contradictory to our in vitro findings according to which EMAP had no direct impact on AsPC-1 proliferation. We think that our previous findings of EMAP blocking the FN induced AsPC-1 proliferation in vitro can in part serve as a plausible explanation. Accordingly, the AsPC-1 proliferative activity may be stimulated or supported within the tumor microenvironment by an FN matrix, a process that EMAP is able to interfere with [31].

Why EMAP did enhance the survival benefits of docetaxel and gemcitabine but not of doxorubicin remains unclear, despite similar observations in local tumor growth and intratumoral proliferation. While it is possible that the antiproliferative mechanism of EMAP is complementary to the cell-cycle-specific actions of taxanes and deoxycytidine analogs but not to those of anthracyclines, this remains speculative. Other possibilities include an agent-specific and variable intratumoral delivery of the cytotoxic drug due to the "antiangiogenic" EMAP effect with increased blood-tumor barrier permeability [38], better tumor perfusion and reduced interstitial pressure [37], or differential drug responses based on EMAP-related changes in HIF-1a expression [35] or TNF receptor upregulation [3].

In summary, our present study demonstrates that EMAP addition increased the antiproliferative and pro-apoptotic

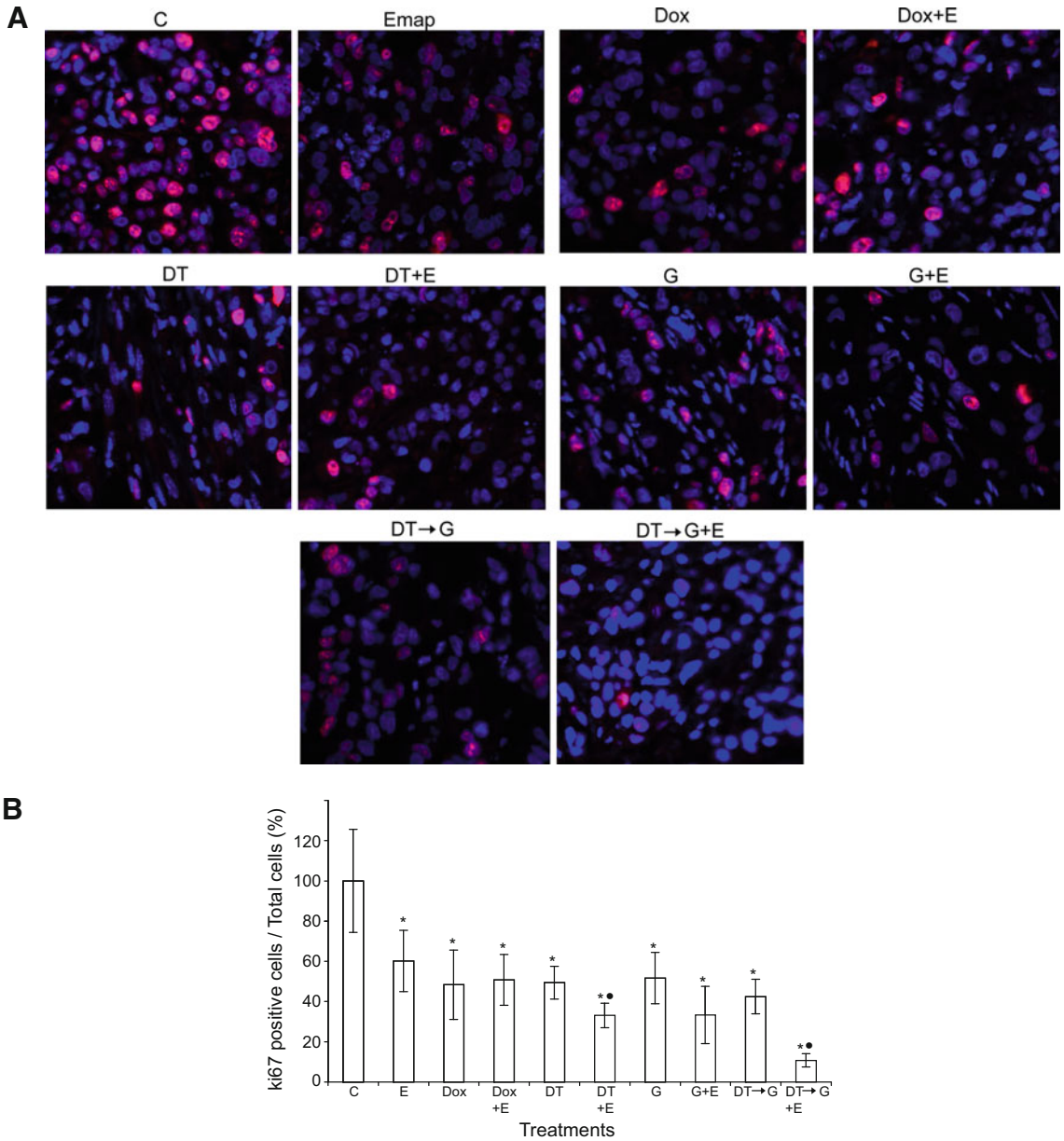

Fig. 5 Effects of doxorubicin (Dox), docetaxel (DT), gemcitabine (Gem), and EMAP (E) treatment on intratumoral proliferative activity. AsPC-1 cells (0.75×10^6) were injected subcutaneously into nude mice. Two weeks after tumor cell injection, mice were treated with PBS (control), Dox, DT, Gem, and E, either alone or in combination, for 3 weeks. Sequential treatment with DT first, and then Gem has been labeled as DT → G. **a** Tumor tissue sections were immunostained with Ki67 nuclear antigen antibody and photographed. **b** Ki67-positive cells were counted per HPF, and the mean counts from five fields were calculated. Data are represented as mean ± S.D. Symbol * represents significant differences ($P < 0.05$) of all treatment groups compared to controls. Symbol • represents significant differences ($P < 0.05$) between the Dox, DT and Gem treatment groups with compared to without EMAP

activity of doxorubicin, docetaxel, and gemcitabine in ECs but not in PDAC cells, while the in vivo benefits seem to be limited to docetaxel and gemcitabine. This is the first report to show that EMAP can enhance antitumor effects of a cytotoxic agent other than gemcitabine. An EMAP-mediated increase in chemotherapeutic drug efficacy therefore not only may enhance clinical benefits of gemcitabine but could render other agents such as taxanes sufficiently effective for clinical applications in PDAC therapy. These findings demonstrate a novel and potentially promising strategy for PDAC treatment that should warrant clinical testing. However, clinical application of EMAP II itself would require a formal drug development process with safety evaluation. An approach utilizing established agents with anti-VEGF and anti-integrin mechanisms in combination with cytotoxic drugs may circumvent this challenge.

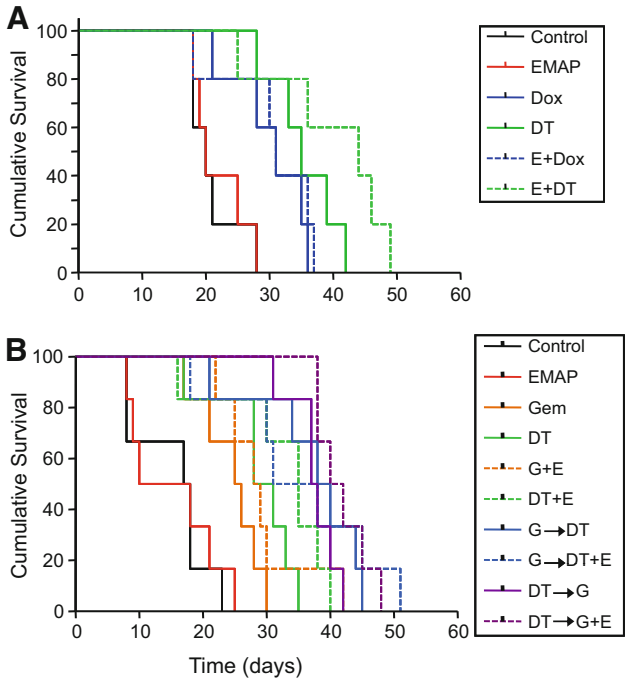

Fig. 6 Effects of doxorubicin (Dox), docetaxel (DT), gemcitabine (Gem), and EMAP (E) treatment on the overall survival of mice in vivo. AsPC-1 cells (0.75×10^6) were injected intraperitoneally in SCID mice and treatment started after 14 days. **a** Mice were treated with PBS (control), Dox, DT, and E, either alone or in combination for 14 days. The curve represents the survival time from the beginning of therapy. The *P* values for survival differences compared to controls are Dox ($P = 0.01$), DT ($P = 0.003$), E + Dox ($P = 0.04$), E + DT ($P = 0.009$). **b** Mice were treated with PBS (control), Gem, DT, and EMAP, either alone or in combination for 21 days. The curve represents the survival time from the beginning of therapy. The *P* values for survival differences compared to controls are Gem ($P = 0.02$), DT ($P = 0.01$), G → DT ($P = 0.003$), DT → G ($P = 0.001$), E + G ($P = 0.003$), E + DT ($P = 0.02$), E + G → DT ($P = 0.005$), E + DT → G ($P = 0.001$)

Conflicts of interest None.

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