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Formulation Comprising Arsenic Trioxide and Dimercaprol Enhances Radiosensitivity of Pancreatic Cancer Xenografts

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Abstract

Objective: To investigate the efficacy of a formula comprising arsenic trioxide and dimercaprol (BAL-ATO) as a radiosensitizing agent in model mice with pancreatic cancer xenografts. **Methods:** Female BALB/c nude mice bearing SW1990 human pancreatic cancer xenografts were divided into four treatment arms, including control, radiotherapy (RT), BAL-ATO, and RT + BAL-ATO groups. Survival and tumor volume were analyzed. We also assessed apoptosis in tumor samples by live imaging and detected hypoxia by confocal laser microscope observation. We further investigated the mechanisms of BAL-ATO action in RT by detecting affected proteins by western blot and immunohistochemistry assays. **Results:** Median survival was significantly longer in the RT + BAL-ATO group (64.5 days) compared with the control (49.5 days), RT (39 days), and BAL-ATO (48 days) groups ($P < 0.001$). RT + BAL-ATO inhibited the growth of tumors in mice by 73% compared with the control group, which was significantly higher than the rate of inhibition following RT alone (59%) ($P < 0.01$). Further analysis showed an improved microenvironment in terms of hypoxia in tumors treated with BAL-ATO alone or RT + BAL-ATO. Expression of signaling molecules associated with pancreatic cancer stem cells, including CD24, CD44, ALDH1A1, Gli-1, and Nestin, was detected in tumors treated with BAL-ATO alone or in combination with RT. **Conclusion:** These data suggest that BAL-ATO function as a radiosensitizer in mice with pancreatic cancer xenografts, via mechanisms involving hypoxia reduction and inhibition of signaling pathways associated with pancreatic cancer stem cells. BAL-ATO may thus be a promising radiosensitizing agent in patients with pancreatic cancer.

Keywords

radiosensitizer, hypoxia, pancreatic cancer stem cells, radiotherapy, arsenic trioxide, dimercaprol

Abbreviations

ATO, arsenic trioxide; BAL, British anti-Lewisite; ESA, epithelial-specific antigen; FBS, fetal bovine serum; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LYM, lymphocyte; PCSC, pancreatic cancer stem cell; PLT, platelet; RBC, red blood cell; RT, radiotherapy; SHH, sonic hedgehog; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; WBC, white blood cell

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Introduction

Pancreatic cancer is the seventh most common cause of cancer deaths, with approximately 330,000 deaths globally each year.[1] Surgery is the most valid therapeutic option for pancreatic cancer; however, most patients with pancreatic cancer are diagnosed with end-stage disease, and only 10%–15% are eligible for surgery. Patients who miss the opportunity to undergo surgery are treated with chemotherapy and/or radiotherapy (RT), and the 5-year survival of patients with pancreatic cancer is currently approximately 10%.[2]

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RT is one of the main treatment methods for pancreatic cancer. However, malignant tumors show different radiosensitivities, and pancreatic cancer is relatively insensitive to RT, with even the highest tolerated dose of irradiation currently incapable of producing an ideal clinical response. It is difficult to achieve satisfactory effectiveness by increasing the dose of external radiation alone, because the pancreas is a retroperitoneal organ that is closely surrounded by normal tissues, such as the liver and intestines.[3] The advantage of RT, either alone or combined with chemotherapy, as a palliative treatment for advanced or relapsed disease is uncertain, and this remedy has so far failed to demonstrate survival benefits in patients with advanced pancreatic cancer.[4]

Arsenic trioxide (ATO) is a traditional, naturally sourced drug for the treatment of acute promyelocytic leukemia worldwide, and has been approved for the treatment of liver cancer in China.[5-7] ATO has previously demonstrated cytotoxic and chemosensitizing effects in pancreatic cancer cells, at least partially by inhibiting the viability of pancreatic cancer stem cells (PCSCs).[8,9] However, ATO has demonstrated less efficacy in clinical trials in patients with pancreatic cancer, and dose-related risks of cardiac and hepatic toxicity limit its clinical application.[10]

Regarding anticancer therapies, several organic arsenics have been designed to produce a response in malignant tumors.[11] Their anticancer mechanisms differ from those of ATO, including their actions on tumor angiogenesis and metabolism and on cell signaling pathways.[12-14] The compound 2,3-dimercaptopropanol (dimercaprol), better known as British anti-Lewisite (BAL), initially synthesized by biochemists at Oxford University approximately a century ago, is still stocked by hospital pharmacies and is occasionally employed in emergencies.[15] Lewisite is a combination of acetylene and arsenic trichloride, and the main use of BAL is to manage Lewisite poisoning by chelating arsenic ions to form nontoxic complexes.[15]

Both ATO and some ligands with active thiols have been studied as radiosensitizers.[16,17] ATO was reported to induce apoptosis in the PCSC subpopulation of pancreatic cancer cells via inhibition of the Sonic hedgehog (SHH) pathway.[8,18] We previously showed that complexes formed by ATO and BAL had similar anticancer effects to ATO, but were less toxic than ATO alone. We therefore hypothesized that the combination of ATO and BAL might reduce the viability of PCSCs and improve hypoxia in pancreatic cancer xenografts in mouse models.

Materials and Methods

Animals, Cell Culture, and Reagents

Five-week-old female athymic nude mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and maintained under specific pathogen-free conditions. The human pancreatic cancer cell line SW 1900 was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). RPMI medium and fetal bovine serum (FBS) were supplied by Gibco Invitrogen (Carlsbad, CA, USA), and cells were maintained in RPMI medium supplemented with 10% FBS. An ATO solution containing 0.9% sodium chloride was provided by Harbin Yida Pharmaceutical Co., Ltd. (China; Lot. 20190102) and 2,3-dimercapto-1-propanol was supplied by Tokyo Chemical Industry Co., Ltd. (Japan; Lot. WG5MB-AT) and stored in anhydrous alcohol. Other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Protocols for animal experiments were approved by the Animal Experimental Ethics Committee of the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University (HKDL2018329), in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Preparation of the Formula

The combination of BAL and ATO was prepared in water, and the resulting chemical structures were examined by mass spectrometry under abiotic conditions. Briefly, an aqueous ATO solution and alcoholic BAL solution were mixed in a tube at a molar ratio of 1:6 (one ATO molecule contains two arsenic ions) and stored at 4°C. The chemical structures were then examined by mass spectrometry (Waters, MA, USA).

Survival and Tumor Volume Analysis

We established mouse tumor models by injecting sub-confluent hormone-independent SW 1990 cells (5×10^6 per mouse) into one flank in each of five mice. Tumor-bearing mice were sacrificed after 3 weeks and the tumors were harvested. Two of the larger tumors were cut into pieces (approximately 2 mm × 2 mm × 2 mm) and then transplanted into mice (n = 48).

The tumor-bearing mice were divided randomly into the following four groups (n = 6 each): control group: injection of saline containing 1% alcohol (v:v); RT group: 1 Gy total body X-radiation plus injection of saline containing 1% alcohol (v:v); BAL-ATO-treated group: injection of freshly prepared BAL-ATO solution; and RT + BAL-ATO group: combined total body RT plus BAL-ATO-treatment. The dose of BAL-ATO was indicated by the quantity of ATO contained in the prepared mixture. The mice were injected daily with intraperitoneal saline or BAL-ATO at a dose equivalent to 30 mg/kg ATO for 5 days within a week.

Xenografted mice were irradiated using an RS 2000 biological system X-ray irradiator (Rad-Source Technologies Inc., Suwanee, GA, USA) (dose rate, 234 cGy/min). RT and RT + BAL-ATO mice received 1 Gy total body irradiation 2 to 4 hours after injection on a Monday, Thursday, and Friday, followed by a second treatment consisting of BAL-ATO alone within a week. The same treatment used in the first week was repeated in the third week.

Survival was observed from the beginning of treatment to the time when all the animals without interventions had died. Tumors were measured every 3 days, and tumor volume was calculated using the following equation: $V = L \times W^2 \times \pi/6$,

where V was tumor volume, L was tumor length, and W was tumor width. Measurements were performed from the first to the 37th day of treatment.

Live Imaging, Hypoxia Analysis, and Sample Collection

Pancreatic cancer xenografted mouse models were established using the methods described above, with six mice in each group. The treatment schedule was the same as described above for tumor-growth analysis.

Three tumor-bearing mice in each group were imaged using an IVIS Lumina II system (Caliper Life Sciences, Hopkinton, MA, USA) equipped with a charge-coupled device camera, 2 days after the last radiation treatment. The mice received intravenous Annexin-vivo 750 (100 μL; catalog no. NEV11053; PerkinElmer, Inc., Waltham, MA, USA) and were then anesthetized with 2% isoflurane in 100% oxygen. Images were acquired by recording bioluminescent signals and analyzed using Living Image software (Version 4.2, Caliper Life Sciences, Hopkinton, MA, USA). After 3 days, the corresponding animals received BAL-ATO treatment 2 hours before administration of HP-RedAPC-MAb (catalog no. HP8-x; Hypoxyprobe, Inc., Burlington, MA, USA). The mice were sacrificed on the 26th day of treatment. Tumors were removed, frozen, and sectioned, and the frozen sections were subjected to hypoxia analysis by confocal laser scanning microscopy (Leica TCS SP8, Leica Microsystems Inc., Wetzlar, Germany).

The remaining mice ($n = 3$) in each group were also sacrificed on day 26 of treatment, and blood samples were drawn from the orbits before euthanasia for analysis using a blood and gas analysis system (KT-6300, Pioway, Nanjing, China). The tumors were harvested and photographed. The removed tumors were separated and kept for analyses by western blot, immunohistochemistry (IHC), terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and hematoxylin and eosin (H&E) staining. Organs including the brain, liver, and kidneys were also removed for H&E staining.

H&E Staining, IHC, and TUNEL Assays

Paraffin-embedded tumor and organ samples were sectioned, placed on slides, dewaxed, rehydrated, pretreated with hydrogen peroxide, washed with phosphate-buffered saline, and then stained with H&E, followed by rinsing and mounting under coverslips using Permount (Fisher Scientific, San Francisco, CA, USA). For IHC staining, endogenous peroxidase was blocked with 3% hydrogen peroxide, and the tissue samples on slides were then incubated with primary anti-human antibodies, including anti-hypoxia-inducible factor (HIF)-1α (catalog no. ab5168; Abcam, Cambridge, UK), anti-CD24 (catalog no. ab31622; Abcam), and anti-CD44 (catalog no. ab189524; Abcam). Color was visualized using an SPlink Detection Kit (catalog no. SP-9000; ZSGB-BIO, Guangzhou, China) in accordance with the manufacturer's instructions. TUNEL assay was performed using an *in situ* cell death detection kit (catalog no. 11684817910; Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. All slides were analyzed and photographed by an experienced pathologist.

Western Blot Analysis

Tumor tissues were washed with cooled phosphate-buffered saline, and samples (about 0.5 cm × 0.5 cm × 0.5 cm) were placed in Eppendorf tubes (Eppendorf, Hamburg, Germany) containing 0.5 mL of lysate solution and kept on ice. The samples were cut into pieces, ground in an electric grinder, and incubated on ice for 30 minutes. Protein was extracted by centrifugation at 15,000 × *g* and quantified with a BCA protein assay kit (Cell Signaling Technology, Inc., Danvers, MA, USA). Protein samples (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 1 hour with 5% non-fat milk solution at room temperature and then incubated with a buffer containing one of the following primary antibodies: anti-HIF-1α (catalog no. ab51608; Abcam), anti-Nestin (catalog no. ab22035; Abcam), anti-Gli-1 (catalog no. sc-515781; Santa Cruz, Santa Cruz, CA, USA), anti-Vinculin (catalog no. 4650S; CST, Danvers, MA, USA), and anti-α-tubulin (catalog no. sc-134237; Santa Cruz) overnight at 4°C. The membranes were then incubated with corresponding peroxidase-labeled anti-mouse (catalog no. P0217; Dako, Glostrup, Denmark) or anti-rabbit (catalog no. P0260, Dako) IgG secondary antibody for 1 hour. Western blot gel images were produced using a Minichemi 610 chemiluminescent imager (Sagecreation, Beijing, China).

Statistical Analysis

Each result was expressed as the mean ± standard error of the mean. Differences between groups were analyzed by one-way ANOVA using PRISM software (GraphPad Software, Inc., San Diego, CA, USA). Significant differences between groups were set at $P < 0.05$.

Results

BAL and ATO Formed Multiple Complexes

We investigated the products of the chemical reaction between BAL and ATO using mass spectrometry. When an alcoholic solution of BAL was added to an aqueous solution of ATO at a molar ratio of 1:6, the solution immediately became creamy white and then became clear after mixing for approximately 5 minutes. Mass spectrometry demonstrated five main peaks, at 342.89, 464.88, 538.79, 660.77, and 782.44 m/z, respectively (Supplemental Figure S1). These peaks were generated by chemical structures capturing a sodium ion (plus 23), indicating that ATO and BAL could form at least five structures in solution (Supplemental Figure S1). After subtracting the atomic weight of sodium, the respective molecular weights were 318.89, 440.88, 515.80, 637.79, and 759.77, and the

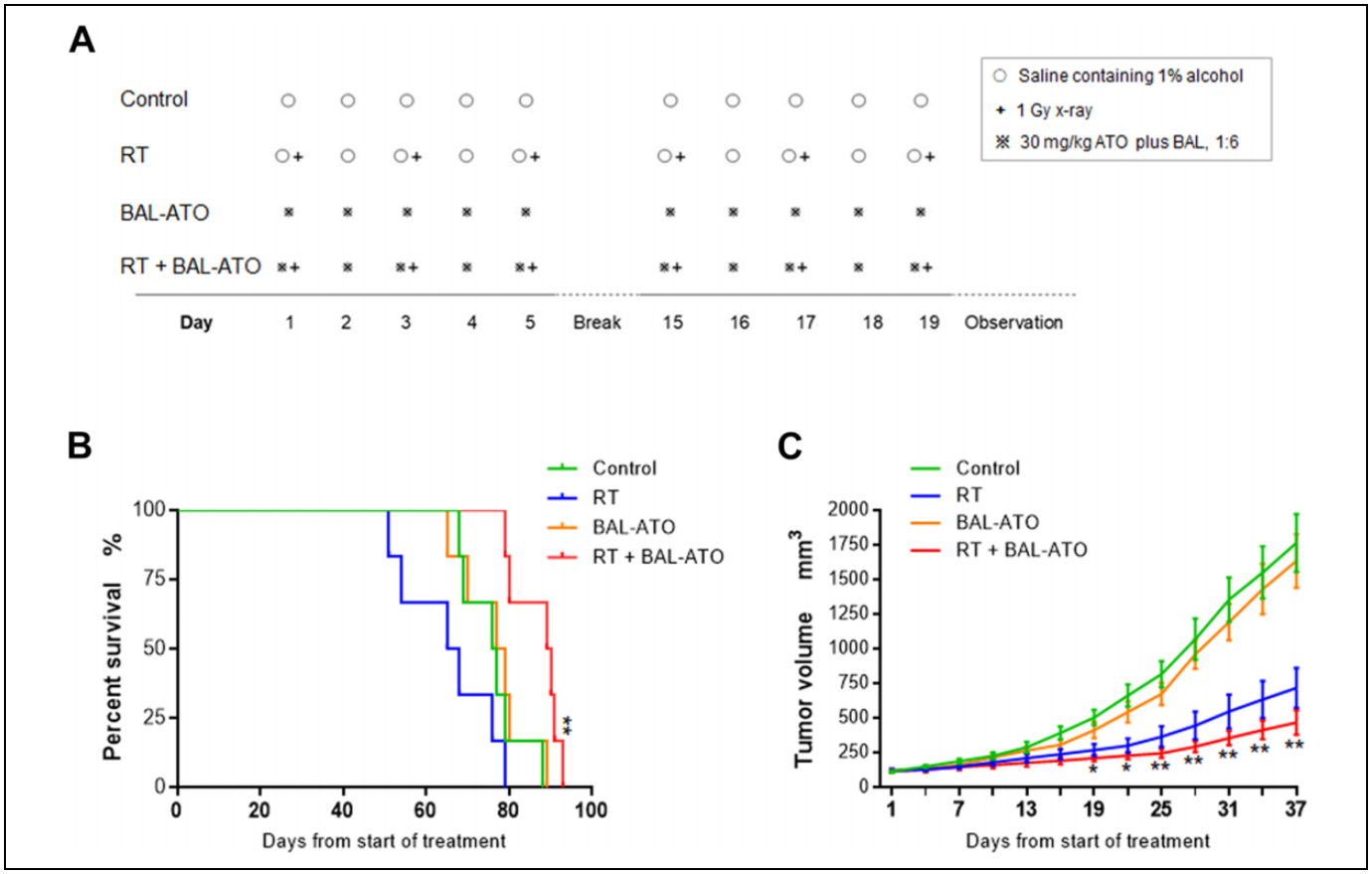

Figure 1. Effects of RT, BAL-ATO, and their combination on mice bearing SW 1990 xenograft tumors. A, Treatment schedule. B, Median survival time (days) was analyzed by Kaplan-Meier survival curves and compared using the log-rank (Mantel-Cox) test. RT + BAL-ATO therapy showed a survival benefit compared with the other groups. $^{**}P < 0.01$ versus control. C, Tumor-growth chart, indicating sensitivity of pancreatic cancer to BAL-ATO and RT. $^{*}P < 0.05$; $^{**}P < 0.01$ versus RT group.

formulas were $C_6H_{12}AsO_2S_4$, $C_9H_{18}AsO_3S_6$, $C_9H_{18}As_2O_3S_6$, $C_{12}H_{24}As_2O_4S_8$, and $C_{15}H_{30}As_2O_5S_{10}$. The mixture containing these ATO/BAL complexes generated by the method described above was labeled as BAL-ATO, and its dose was calculated according to the quantity of ATO contained.

BAL-ATO Enhanced the Radiation-Induced Inhibition of Tumor Growth

We evaluated the radiosensitizing effect of BAL-ATO *in vivo* in 24 SW 1990 xenograft-bearing mice (Figure 1A). The *in vivo* therapeutic effects of BAL-ATO and RT were evaluated by survival analysis. Mice treated with RT + BAL-ATO (64.5 days) survived significantly longer (median 64.5 days) than mice in the control (49.5 days), BAL-ATO (48 days), and RT (39 days) groups ($P < 0.001$, Figure 1B). There was no significant difference in survival duration between the control and BAL-ATO treatment groups (Figure 1B). In addition, about 70% of mice in the RT group had mouth ulceration, diarrhea, and weight-loss, compared with about 30% of mice in the RT + BAL-ATO group, indicating a lower incidence and slighter symptoms in the latter group.

Changes in tumor volumes in the different treatment groups were examined pathologically within 37 days. At 37 days, tumors in the RT + BAL-ATO group (469 ± 89 mm^3) were significantly smaller than those in the RT alone (719 ± 144 mm^3, $P < 0.01$), BAL-ATO (1636 ± 193 mm^3, $P < 0.01$), and control groups (1765 ± 203 mm^3, $P < 0.01$). BAL-ATO significantly enhanced the radiation-induced inhibition of tumor growth in the mouse models. RT + BAL-ATO inhibited the growth of tumors in mice by 73% compared with the control group, which was much higher than the rate of inhibition following RT alone (59%), with an increase in tumor-doubling time from 17 to 25 days (Figure 1C, $P < 0.01$). The data also demonstrated that, although BAL-ATO inhibited tumor growth during treatment, tumor size still increased with increasing time after drug administration.

Apoptosis imaging in live animals is a useful tool for evaluating the effects of anticancer treatments. We therefore assessed the effects of BAL-ATO and RT in three mice from each group by live-imaging after injection of Annexin-vivo 750 on day 22. The luminescence intensity was strongest in mice in the RT + BAL-ATO group (Figure 2A).

The remaining three mice in each group that did not undergo live imaging were euthanized on day 26 and the tumors were

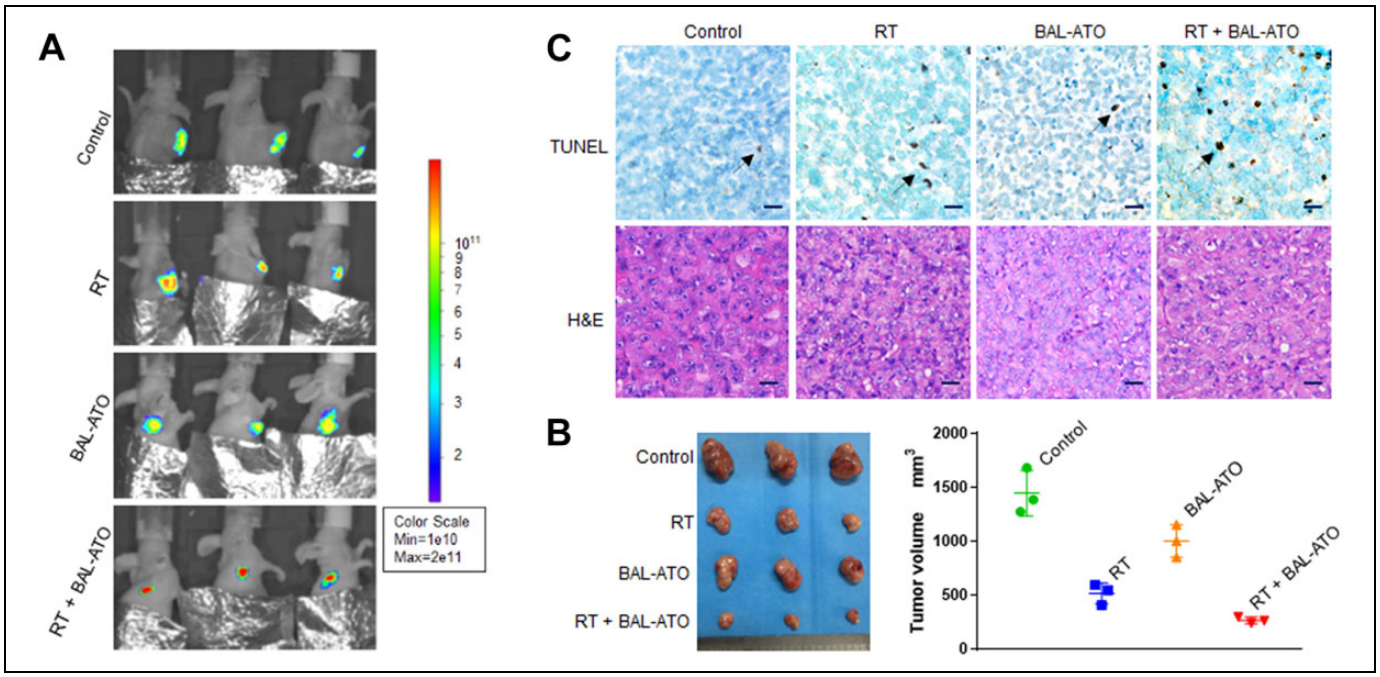

Figure 2. BAL-ATO facilitated RT-induced apoptosis in pancreatic cancer xenografts. Mice with tumor xenografts were treated as shown in Figure 1A. A, Three mice in each group underwent live imaging after Annexin-vivo 750 injection on day 22. Mice in the RT + BAL-ATO group showed the strongest fluorescence intensity among the groups. B, Three mice from each group that did not undergo live imaging were euthanized, and tumor samples were harvested on day 26. C, Tumors from mice in the RT + BAL-ATO group showed more positive-stained cells in TUNEL assay (Supplemental Table S1), and more apoptotic cell characters, such as nuclear shrinkage or breakage, by H&E staining. Scale bars, 20 μm.

harvested for TUNEL and H&E staining assays. Variations in tumor volume were observed among the different groups (Figures 1C and 2B). These differences were consistent with the histopathological differences. Tumors from mice in the RT + BAL-ATO group showed more TUNEL-positive cells compared with the other three groups (Figure 2C and Supplemental Table S1), and more cell-death characteristics, such as cellular shrinkage and nuclear fragmentation, in H&E-stained tissue sections (Figure 2C). Taken together, these data indicated that the combination of RT + BAL-ATO was more effective than RT alone for inhibiting tumor growth in pancreatic cancer xenograft-transplanted mice.

BAL-ATO Reduced Hypoxia in Pancreatic Cancer Xenografts

Mice that underwent live imaging were subsequently euthanized after injection with HP-RedAPC (Hypoxyprobe, Inc.), according to the protocol supplied with the kit, on day 26. The tumors were harvested and hypoxic conditions were examined under a laser confocal microscope. Tumors from mice treated with RT showed stronger staining or more positive cells, indicating that RT increased tumor hypoxia. However, red staining was reduced in tumors in the BAL-ATO and RT + BAL-ATO groups, indicating that BAL-ATO improved the hypoxic microenvironment in irradiated tumors (Figure 3A).

Tumor samples harvested from the mice that did not undergo live imaging on day 26 were prepared for IHC and western blot assays using HIF-1α antibody. Downregulation of HIF-1α protein was detected in the BAL-ATO and RT + BAL-ATO groups by both IHC (Figure 3B and Supplemental Table S1) and western blot (Figure 3C), consistent with the trend in hypoxia observed in frozen tissue sections using laser confocal microscopy.

BAL-ATO Treatment Was Associated With PCSC Signaling Pathways

We examined the relationship between BAL-ATO treatment and PCSCs by biochemical analysis of tumor samples harvested from three mice in each group that did not undergo live imaging. Expression of the cell surface markers CD44, CD24, and epithelial-specific antigen (ESA) is frequently considered to be characteristic of PCSCs.[19] Increased expression levels of CD24 and CD44 were detected in the RT group by IHC assay; however, CD24 and CD44 expression both declined in the BAL-ATO and RT + BAL-ATO groups, indicating that BAL-ATO alone or in combination with RT weakened the characteristics of PCSCs (Figure 4A and Supplemental Table S1). Gli-1, ALDH1A1, and Nestin proteins have also been

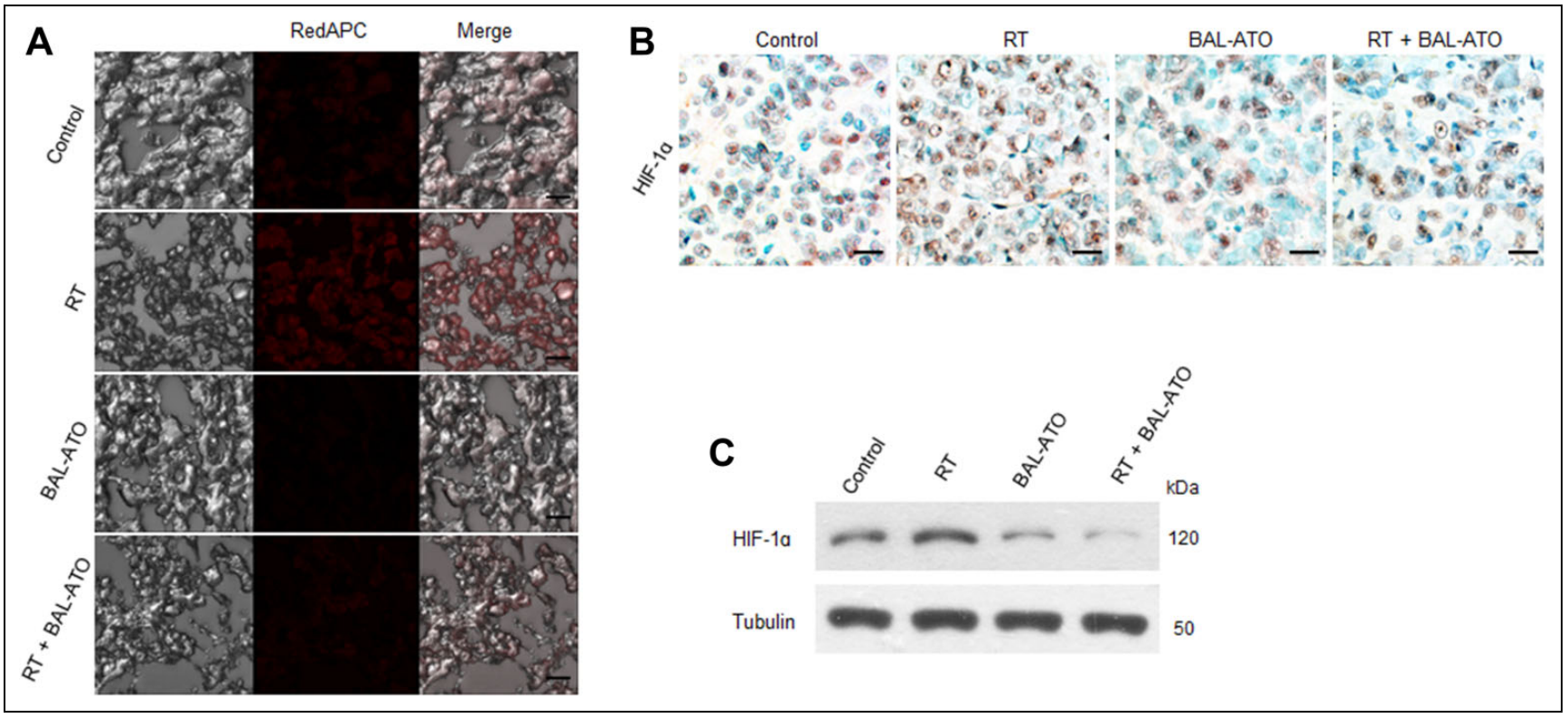

Figure 3. BAL-ATO improved the hypoxic microenvironment in pancreatic cancer xenografts. A, Mouse models (n = 3; as described in Figure 2A) were injected with HP-RedAPC-MAb on day 26 and the tumors were harvested, prepared for frozen tissue sections, and observed under a confocal laser microscope. Tumors treated with RT showed the strongest red staining, and tumors treated with RT + BAL-ATO showed weaker staining. B, IHC assay of tumors (n = 3; as described in Figure 2C) for HIF-1α. Tumors from the RT group showed more and more strongly HIF-1α-positive cells than the other groups. There was no significant difference between tumors from the RT + BAL-ATO and control groups (see Supplemental Table S1). C, Extracts from tumors (as described in Figure 2C) were subjected to western blot analysis with anti-HIF-1α human antibody. Tumors in the RT + BAL-ATO group showed significantly lower HIF-1α expression than tumors in the RT group ($P < 0.01$).

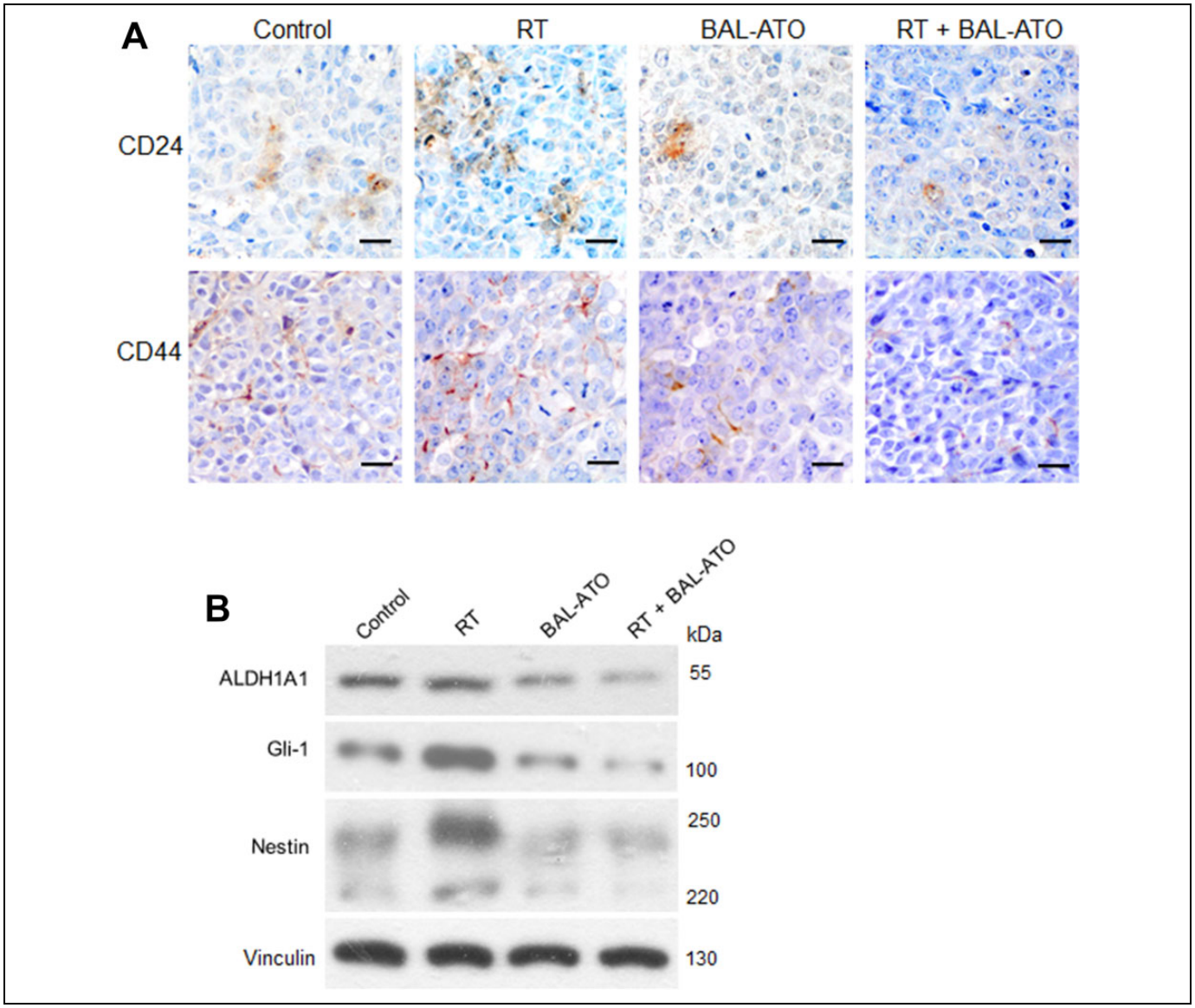

Figure 4. BAL-ATO treatment reduced the PCSC characteristics in pancreatic cancer xenografts. IHC and western blot assays of tumor samples (n = 3; as described in Figure 2C). A, CD24 and CD44 expression levels were lower in the RT + BAL-ATO group compared with the other groups. Scale bars, 20 μm. B, Expression levels of proteins associated with PCSCs, including ADLH1A1, Gli-1, and Nestin, were downregulated in the BAL-ATO and RT + BAL-ATO groups, according to western blotting.

Table 1. Effects of ATO-BAL on Blood Cells in Irradiated Mice × 10^9/L.

	Control	RT	BAL-ATO	RT + BAL-ATO
WBC	16.3 ± 1.3	29.3 ± 4.9*	17.9 ± 1.2	18.5 ± 0.8
RBC	7.2 ± 0.3	7.3 ± 0.4	6.7 ± 1.6	7.4 ± 0.4
PLT	313.3 ± 18.4	323.0 ± 42.5	280.0 ± 24.7	278.7 ± 10.6
LYM	6.6 ± 0.4	10.2 ± 1.7*	7.1 ± 0.5	6.3 ± 0.5

Blood samples were collected from the orbit before euthanasia and examined immediately (n = 3).
*$P < 0.01$ versus control.

associated with PCSCs.[20] Western blot assay detected downregulation of these proteins in the BAL-ATO and RT + BAL-ATO groups compared with the control (Figure 4B). Taken together, IHC and western blot analyses indicated that BAL-ATO could reduce the characteristics of PCSCs, which might play an important role in enhancing the killing effect of X-rays on pancreatic cancer cells *in vivo*.

BAL-ATO Protected Tumor-Xenografted Mice From Radiation Injury

Radiation can damage the immune system, lead to bacterial infections, and can increase the white blood cell (WBC) count, which is a sensitive indicator of infection.[21] In addition to survival time, we also investigated the protective effects of BAL-ATO administration. Routine blood tests revealed more WBCs in the RT group compared with the control group ($P <$

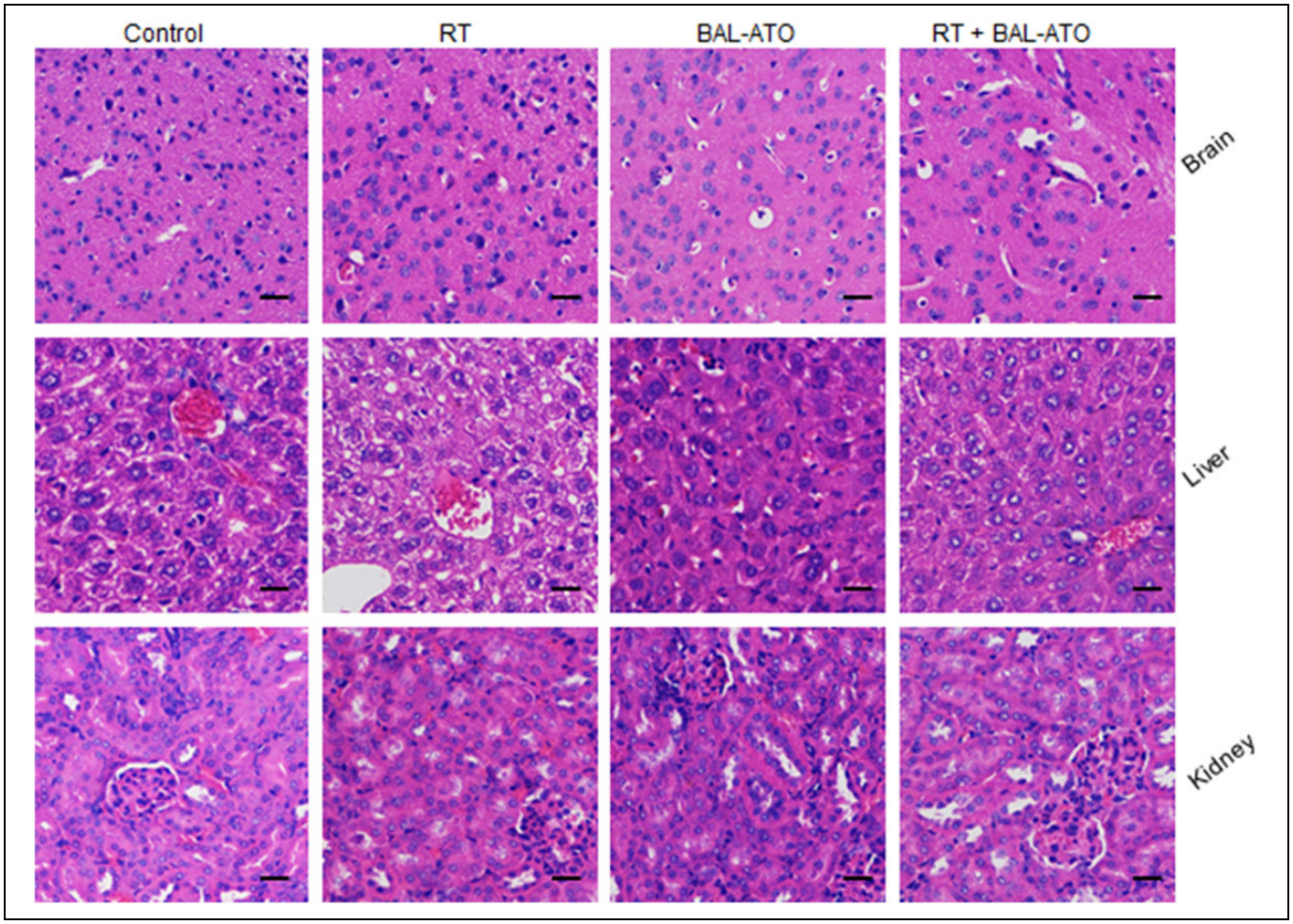

Figure 5. BAL-ATO protected against X-irradiation-induced tissue damage. Brain, liver, and kidney samples were obtained from mice (n = 3, as described in Figure 2C) and subjected to H&E staining. Scale bars, 25 μm.

0.01, Table 1); however, there was no significant difference between the RT + BAL-ATO and control groups (Table 1), indicating that BAL-ATO might protect animals from radiation-induced impairment of immunity. We also removed liver, brain, and kidneys from mice in each group for H&E staining. Liver sections in the RT group showed alterations including cell swelling and nuclear disruption, but these changes were not seen in the other three groups (Figure 5). Differences in brain and kidney tissues were less obvious (Figure 5), indicating that X-irradiation might mainly affect the liver, and that BAL-ATO might protect this organ from injury.

Discussion

ATO is a traditional natural drug used to treat some malignant diseases in China. Emerging evidence has suggested that ATO could be employed as a radiosensitizer; however, its clinical translation is limited by its evident toxicity.[22-24] We previously found that ATO demonstrated modest inhibition of tumorigenesis in pancreatic cancer xenografts, while dose-related risks of cardiac and hepatic toxicity have frequently been reported by clinical investigators.[8] We examined the abilities of drugs that chelate arsenic cations (As^{3+}) to form complexes, to improve the anticancer effects of ATO, especially against pancreatic cancer. Among those drugs, we showed that BAL which was designed by British biochemists as an antidote for the now-obsolete arsenic-based chemical warfare agent, Lewisite, during World War II, facilitated pancreatic cancer cell killing by ATO.

We performed preliminary Cell Counting Kit-8 tests to evaluate the cytotoxicity of the drugs prior to the animal experiments, and BAL showed no obvious inhibition in cell cultures at concentrations up to 30 μM (see Supplementary File S1). BAL-ATO significantly inhibited the proliferation of pancreatic cancer cells *in vitro*, either with or without X-irradiation; however, the effects of BAL-ATO on tumor growth without X-irradiation has not been observed *in vivo*, and we therefore assessed the function of BAL-ATO as a radiosensitizer against pancreatic cancer in the current study.

We initially evaluated the systemic toxicity of the drugs in five mice, and showed that BAL-ATO had less systemic toxicity than either ATO or BAL alone. Mice could tolerate

30 mg/kg ATO along with the corresponding quantity of BAL (ATO:BAL = 1:6), but could not tolerate either monotherapy, and neither ATO nor BAL alone was therefore included in this study. Meanwhile, the current study showed that injection of BAL-ATO slightly inhibited the tumorigenesis of pancreatic cancer xenografts, but BAL-ATO combined with low-dose X-irradiation resulted in significant growth inhibition of pancreatic cancer xenografts. Compared with radiation alone, RT + BAL-ATO prolonged the tumor doubling-time approximately two-fold. However, analysis of blood samples and organs, including the brain, liver, and kidneys, demonstrated that BAL-ATO might reduce injury resulting from X-irradiation.

Hypoxia occurs in most solid tumor tissues and is considered to be a major cause of radioresistance.[25,26] Most radiosensitizers currently consist of compounds containing nitro groups, such as misonidazole and RRx-001.[27-29] Although these drugs increase the anticancer effects of RT compared with RT alone, their obvious toxicity and adverse effects limit their clinical applications. ATO is also being studied as a radiosensitizer.[24,30] In this study, we evaluated the ability of a formula containing ATO and BAL to reduce the toxicity of ATO by forming multiple complexes, and investigated its suitability as a radiosensitizer *in vivo*. The results of laser confocal microscopy, IHC, and western blot analyses showed that BAL-ATO improved hypoxia in the tumor microenvironment, even in tumors that had undergone irradiation. Furthermore, our results suggested that tumor hypoxia might be aggravated by irradiation, while BAL-ATO might alleviate the worsening condition within tumors.

Increasing evidence supports the existence of human PCSCs, and treatments targeting these cells have been suggested as a strategy to kill cancer cells completely.[31] The subpopulation of PCSCs, defined by expression of the cell surface markers $CD44^+CD24^+ESA^+$, comprises approximately 0.2%–0.8% of all cells in tumor tissues.[8,32] The SHH signaling pathway plays a critical role in the survival and proliferation of tissue stem and progenitor cells, and SHH is markedly upregulated in $CD24^+CD44^+ESA^+$ pancreatic cancer cells compared with $CD24^-CD44^-ESA^-$ and bulk cells.[33] Previous studies proposed that the activity of PCSCs could be reduced by ATO via binding to Gli proteins, as members of the SHH pathway.[8] In this study, IHC and western blotting demonstrated that BAL-ATO downregulated the expression of Gli-1 proteins, while ALDH1A1 and Nestin proteins, as indicators of stem cells, were also downregulated in BAL-ATO-treated tumors according to western blotting. Moreover, CD24 and CD44 expression levels were evidently decreased in tumors from mice treated with BAL-ATO or RT + BAL-ATO, indicating that BAL-ATO might suppress the viability of PCSCs.

Some compounds, such as disulfiram, have thiol groups, and their metal complexes have been reported to act as radiosensitizers in a range of cancers via their potential to induce oxidative stress.[34] Like disulfiram, BAL includes thiol groups and has the capacity to form multiple complexes with arsenic cations. The results of the current study suggest that the BAL-ATO formulation enhanced the effects of radiation against pancreatic cancer *in vivo*. Furthermore, the radiosensitizing mechanism of BAL-ATO was associated with changes in the hypoxic microenvironment and suppression of PCSC viability.

This study had some limitations. Notably, the chemical composition of BAL-ATO formula contained multiple complexes, and free BAL molecules as well as arsenic cations, then further screening tests are therefore needed to identify the most effective molecules with benefits in RT.

In summary, the prepared BAL-ATO formulation demonstrated significant activity to improve radiosensitiviy with tolerable systemic toxicity in a mouse model of pancreatic cancer, suggesting that it might be a useful candidate radiosensitizer in patients with pancreatic cancer

Authors' Note

Renyan Tang, MD, and Jianmin Zhu, MD, contributed equally to this work. Jinbin Han and Jianmin Zhu made substantial contributions to the design of the study plan, selection protocol and the design of the methods used in this study. Renyan Tang, Ning Wu and Ying Liu performed the experiments. Jinbin Han and Renyan Tang wrote as well as revised this article. All authors contributed significantly to the manuscript, read the manuscript, gave final approval for the version to be published, and take public responsibility for appropriate portions of the content. Protocols for animal experiments in this study were approved by the Animal Experimental Ethics Committee of the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University (HKDL2018329).

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Declaration of Conflicting Interests

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Supplemental Material

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