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Enhanced anti-cancer effect of AMTB hydrochloride via chitosan nanoparticles in pancreatic cancer

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Abstract

Pancreatic cancer is a malignancy with poor prognosis and high mortality. This study investigated the use of chitosan nanoparticles (CS-NPs) to encapsulate AMTB, a TRPM8 inhibitor, as a novel strategy to enhance therapeutic efficacy in pancreatic cancer. TRPM8 was overexpressed in pancreatic cancer tissues and associated with poor patient prognosis. AMTB inhibited pancreatic cancer cell proliferation, migration, and invasion by suppressing the EMT process and MMP2/9 expression. CS-NPs@AMTB were successfully synthesized, exhibiting excellent drug release profiles and stronger anti-tumor effects than free AMTB. Both AMTB and CS-NPs@AMTB demonstrated favorable biological safety. This is the first study to apply chitosan nanoparticles for AMTB delivery in pancreatic cancer, significantly enhancing its anti-tumor and anti-metastatic effects (ahout 70% reduction in tumor size). These findings suggest that CS-NPs@AMTB might overcome current therapeutic limitations by improving drug efficacy and targeting metastasis in pancreatic cancer. With further validation through preclinical and clinical studies, this nanoparticle-based delivery strategy holds promise for integration into future therapeutic regimens and personalized treatment approaches.

Keywords Pancreatic cancer, AMTB, Chitosan, Nanoparticle, TRPM8, Lung metastasis

Introduction

Pancreatic cancer is a devastating malignancy with a limited survival rate, with only 5–7% of patients surviving more than 5 years after diagnosis [1]. Despite recent advancements in treatments such as radiotherapy and chemotherapy, pancreatic cancer patients' overall survival rate remains low because of high resistance of pancreatic cancer cells to these therapies. The only curative treatment course remains surgical resection [2]. Recent innovations in drug delivery systems, such as liposomes, nanoparticles, and carbon nanotubes, have shown promise in improving the therapeutic efficacy of cancer treatments in pre-clinical trials. However, enhancing drug specificity, stability, and targeted delivery to tumor sites remains a significant challenge [3, 4]. Therefore, the development of novel, specific, tumor-targeted drug delivery systems is crucial for improving the treatment outcomes of this aggressive disease.

Transient receptor potential channels (TRP channels), a superfamily of ion channels, play vital roles in various physiological processes, including thermoregulation, pain transmission, vasodilation, and tumorigenesis [5]. Among the TRP channels, TRPM8 has gained attention due to its involvement in regulating cell proliferation, migration, apoptosis, and inflammation [6]. Moreover, TRPM8 is aberrantly expressed in multiple tumors [7],

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including colorectal cancer [8], hepatocellular cancer [9], and bladder cancer [10]. Notably, TRPM8 is also overexpressed in pancreatic cancer, where it contributes to tumor progression by promoting proliferation and metastasis [11–13], making it a potential therapeutic target. TRPM8 inhibitors have demonstrated anti-tumor effects across several malignancies [14]. Among these inhibitors, AMTB hydrochloride, a 2-benzyloxy-benzoic acid amide derivative, has been used in several studies to block TRPM8 activity [15, 16]. AMTB has shown efficacy in suppressing tumor growth and metastasis in osteosarcoma, prostate, and breast cancers [17–19]. Compared to other TRPM8 inhibitors like menthol and camphor derivatives, AMTB exhibits higher specificity for TRPM8, targets multiple tumor-related pathways, and shows low toxicity, making it a promising candidate for pancreatic cancer therapy [17, 20, 21]. Although RQ-00203078, another TRPM8 inhibitor, is a selective and orally active TRPM8 antagonist [22], its anticancer potential has only been investigated in oral squamous carcinoma cell lines [20] and esophageal cancer cells [23]. In contrast, AMTB has already shown promising antitumor activity in multiple cancer types [18, 19, 24], supporting its selection for investigation in pancreatic cancer. However, its potential role in pancreatic cancer remains unexplored.

Chitosan (CS), a polysaccharide derived from chitin through deacetylation, is known for its biocompatibility, biodegradability, and low toxicity, making it an ideal candidate for drug delivery applications [25]. Compared to other nanoparticle-based carriers such as liposomes and PEGylated nanoparticles, chitosan nanoparticles offer several distinct advantages, including mucoadhesiveness, enhanced cellular uptake through electrostatic interactions with negatively charged cell membranes, and the ability to open tight junctions to improve paracellular transport [26–28]. Additionally, CS-NPs are easily modifiable for ligand attachment, exhibiting lower immunogenicity [29]. These properties make CS-NPs particularly suitable for delivering hydrophobic anticancer agents like AMTB in the context of pancreatic cancer. Chitosan nanoparticles (CS-NPs) enhance the efficacy of cancer treatments due to their large size, surface charge, and diverse morphology [30]. These nanoparticles act as carriers and bind to various biomolecules, contributing to enhanced drug stability and transportation. For instance, CS-NPs have been shown to enhance the anti-cancer activity of Morinda citrifolia essential oil with minimal cytotoxicity [31]. Additionally, CS-NPs loaded with gemcitabine enhanced apoptotic and ferroptotic responses in pancreatic cancer cells [32], while chrysin-loaded CS-folic acid nanoparticles suppressed pancreatic cancer cell growth through apoptosis and angiogenesis inhibition [33].

In this study, we encapsulate the TRPM8 inhibitor AMTB hydrochloride in CS-NPs to enhance its anticancer effect in pancreatic cancer. We aim to investigate the therapeutic potential of the CS-NPs@AMTB system through in-vitro and in-vivo experiments. This study is the first to investigate AMTB hydrochloride as a treatment for pancreatic cancer and to develop a chitosan nanoparticle-based delivery system for its targeted application. This approach offers a promising and novel strategy for overcoming the limitations of current pancreatic cancer therapies.

Materials and methods

A flowchart of the experimental design is provided in Supplementary Figure S1 and the experimental methods are detailed in Supplementary Materials and Methods.

Cell lines

Human in situ pancreatic carcinoma cell lines BxPC-3, PANC-1, AsPC-1, and PATU-8988 and human normal pancreatic ductal epithelial cells HPDE6-C7 (BNCC359453) and mouse pancreatic adenocarcinoma cell line Panc02 (BNCC338034) were applied in the study.

MTT assay

Cell viability of BxPC-3 and PANC-1 cells were assessed using the MTT assay upon different concentrations (10, 20, and 30 μM) or (0, 2, 4, 8, 16, and 32 μM) of AMTB (HY-100345, MCE, Shanghai, China) [19, 24].

Colony formation assay

Cell proliferation of BxPC-3 and PANC-1 cells were detected using the colony formation assay.

EdU staining

Cellular DNA synthesis was detected using Cell-Light EdU Apollo567 in Vitro Kit (C10310-1, Ribobio, Guangzhou, China).

Scratch test

Cell migration ability of BxPC-3 and PANC-1 cells were ascertained using the scratch test.

Transwell assay

Cell invasion ability of BxPC-3 and PANC-1 cells were determined using the Transwell assay.

Western blot

Western blot was carried out to detect TRPM8, E-cadherin, N-cadherin, Snail, MMP2 and MMP9 expression within pancreatic cancer tissue and cells. The relevant primary antibodies are as follows: GAPDH (5174 S, 1:1000, Cell signaling Technology [CST], Boston, USA), TRPM8 (ab85617, 1:1000, Abcam), E-cadherin

(20874-1-AP, 1:20000, Proteintech, Wuhan, China), N-cadherin (AF4039, 1:500, Affinity Bioscience), Snail (CSB-PA004123, 1:1000, CUSABIO, Wuhan, China), MMP2 (CSB-PA003258, 1:1000, CUSABIO), and MMP9 (ab283575, 1:1000, Abcam).

Synthesis of CS-NPs@AMTB

CS-NPs were chosen as the delivery system for AMTB due to their favorable characteristics, including biocompatibility, biodegradability, and ability to efficiently encapsulate hydrophobic drugs like AMTB. Additionally, CS-NPs have been widely used in drug delivery systems for their potential to enhance cellular uptake and provide sustained release, which is essential for overcoming the challenges of targeting pancreatic cancer cells. The synthesis, characterization, and application of nanoparticles in this study adhere to the guidelines established by the National Technical Committee on Nanotechnology of Standardization Administration of China, ensuring responsible conduct in the design, execution, and reporting of research with nanomaterials. CS-NPs were synthesized by ionotropic gelation using tripolyphosphate (TPP) as a crosslinking agent.

Characterization of CS-NPs@AMTB

Transmission electron microscopy (TEM, FEI Talos F200X, Thermo Fisher Scientific) was used for the morphological examination of CS-NPs and CS-NPs@AMTB. The measurement of the particle size distribution and nanoparticle stability of CS-NPs@AMTB was performed by Dynamic light scattering (DLS).

In-vitro drug release calculations

The nanoparticles were dissolved in 10 mL PBS to separately conduct the AMTB release from CS-NPs@AMTB (5 mg) studies at pH 5.0, pH 6.5, and pH 7.4.

Determination of encapsulation efficacy (EE)

Freshly prepared CS-NPs@AMTB were centrifuged at 15,000 rpm for 30 min. The absorbance of the supernatant was determined using UV/visible spectroscopy. The concentration of AMTB was estimated using the AMTB standard calibration curve.

Hemolysis rate

Hemolysis rate assay was applied to determine the biocompatibility of CS-NPs@AMTB on blood cells.

Cellular uptake assay of CS-NPs@AMTB

Cellular uptake assay was used to evaluate the efficiency of cellular uptake of CS-NPs@AMTB.

In vivo studies

All animal experiments were conducted in accordance with the institutional guidelines for animal care and use, and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Fourth Hospital of Changsha. Animal welfare was closely monitored throughout the study. Mice were weighed daily, and signs of distress or illness, such as weight loss greater than 20% or unresponsiveness, were used as criteria for humane endpoints, at which point animals were euthanized by cervical dislocation after 2% isoflurane anesthesia. Efforts were made to minimize animal suffering, and all procedures were performed under anesthesia. A sample size of 6 mice per group was chosen based on previous studies in similar experimental models [34]. Twenty-four 4-week-old male BALB/c nude mice (weighing 16.0 ± 3.0 g) and twenty-four 6-week-old male C57BL/6 mice (weighing 20.0 ± 3.0 g) were procured from Hunan SLAC laboratory animal Co. ltd. (Changsha, China). A tumor xenograft model in BALB/c nude mice was employed to assess the therapeutic potential of CS-NPs@AMTB on the growth of pancreatic cancer cells. The lung metastasis model in C57BL/6 mice was applied to investigate the potential of CS-NPs@AMTB in preventing metastasis in pancreatic cancer cells.

In vivo safety evaluation of CS-NPs@AMTB

The in vivo safety evaluation of CS-NPs@AMTB provided the biocompatibility, potential toxicity, and safety profile of CS-NPs@AMTB in living organisms of C57BL/6 mice, including heart, liver, spleen and kidney.

TUNEL staining

Tumor apoptosis was evaluated using TUNEL staining.

Clinical samples

Pancreatic cancer and adjacent non-cancerous tissue samples (where adjacent non-cancerous tissues were defined as tissue more than 2 cm away from the tumor [35]) from 30 patients with pancreatic cancer were collected in this study. These specimens were obtained from subjects undergoing radical surgical resection of pancreatic cancer from January 2022 to January 2023 at the Fourth Hospital of Changsha. The clinical and pathologic characteristics of pancreatic cancer patients are listed in Table S1. All patients included in the study had not undergone other treatments such as radiotherapy, chemotherapy, or targeted therapy prior to surgery. All pancreatic cancer and adjacent non-cancerous tissues were confirmed by pathologists.

HE staining

H&E staining was applied to evaluate the morphology and structure of pancreatic cancer tissues.

IHC staining

IHC staining was used to visualize and ascertain TRPM8 and Ki-67 proteins in pancreatic cancer tissues. The relevant primary antibodies are as follows: TRPM8 (DF7966, Affinity Bioscience, Changzhou, China), Ki-67 (ab15580, Abcam, Cambridge, USA).

qRT-PCR

qRT-PCR was carried out to detect TRPM8 expression within disc pancreatic cancer tissues and cells. GAPDH was used for internal reference. which was calculated using the $2^{-\Delta\Delta Ct}$ method. The detailed amplification primer sequences for each gene and its internal control are provided in Table 1.

Statistical analysis

The cell experiments were performed at least thrice. The animal experiments were performed at least six times. All experimental data were processed using SPSS 21.0 statistical software (IBM, Armonk, USA) and presented in the forms of mean ± standard deviation of the results obtained from at least three independent experiments. Data were analyzed by an investigator blinded to the procedure performed. The Kolmogorov–Smirnov test assessed whether the data were in normal distribution. A student's *t*-test was employed to compare the differences between the two groups. Differences among three groups or above were estimated using one-way ANOVA, followed by the LSD test or Dunnett T3 test. Data that were not normally distributed were analyzed using the Kruskal-Wallis test. The significance level was set at $P < 0.05$.

Results and discussion

TRPM8 antagonist AMTB inhibits pancreatic cancer cell proliferation

After treating the cells with graded concentrations of AMTB (a TRPM8 antagonist), MTT, colony formation and EdU assays were conducted to assess the changes in cell proliferation. The results suggested that the proliferative capacity of AMTB-treated cells was significantly suppressed dose-dependently (all $P < 0.05$, Fig. 1A-D). Specifically, the cell proliferation was decreased by approximately 80% at the highest concentration of AMTB compared to the control group. These results collectively imply that AMTB could repress pancreatic cancer cell proliferation. Similar to our findings, previous studies have also shown the inhibitory effect of AMTB on tumor cell proliferation. AMTB decreases viable cell number in breast cancer cells [19]. AMTB resulted in suppressed proliferation and apoptosis induction in osteosarcoma cells [17].

Table 1 List of the primers utilized for qRT-PCR

Name of primer		Sequence (5′-3′)
TRPM8	Forward	CTCTTTGTATTCTGGACGAGTCATTT
	Reverse	TCTTGGGTCCTAAGTTTCTGCT
GAPDH	Forward	ACAGCCTCAAGATCATCAGC
	Reverse	GGTCATGAGTCCTTCCACGAT

TRPM8 antagonist AMTB restricts pancreatic cancer cell migration and invasion

The different concentrations of AMTB treatments dramatically suppressed pancreatic cancer cell migration (all $P < 0.05$, Fig. 2A) and invasion (all $P < 0.05$, Fig. 2B) concentration-dependently. Specifically, the migration was reduced by 65% ($P < 0.01$, Fig. 2A), and invasion was reduced by 50% ($P < 0.01$, Fig. 2B) in cells treated with the highest concentration of AMTB. Subsequently, the related mechanism of AMTB affecting the biological functions of pancreatic cancer was also preliminarily explored, followed by the testing of the expression levels of epithelial-to-mesenchymal transition (EMT)-related proteins and invasion and migration-associated genes MMP2 and MMP9. The findings indicated that gradient concentrations of AMTB markedly promoted E-cadherin expression and suppressed N-cadherin, Snail, MMP2, and MMP9 expression (all $P < 0.01$, Fig. 2C). These results reveal that AMTB attenuates pancreatic cancer cell metastasis by inhibiting EMT and MMP2/9 expression levels.

Herein, the suppressive effect of AMTB on EMT was reported for the first time. EMT is the deadliest feature of cancer, resulting in metastasis and chemoresistance [36]. Therefore, EMT has become a key target in the treatment of malignant tumors. Previous literature has reported that TRPM8 can indirectly promote EMT by upregulating Wnt5a [37]. The inhibition of EMT by TRPM8 antagonism, as seen with AMTB, may be attributed to several molecular pathways. One key pathway is the regulation of the Wnt/β-catenin signaling pathway, which has been reported to be activated by TRPM8 in colorectal cancer [8]. TRPM8 inhibition could reduce the nuclear translocation of β-catenin, thereby repressing the expression of mesenchymal markers like N-cadherin and Snail, while promoting the expression of the epithelial marker E-cadherin. Moreover, the PI3K-Akt signaling pathway, known to be activated by TRPM8 [38], could also contribute to the regulation of EMT. Inhibition of TRPM8 may disrupt this pathway, further enhancing the epithelial phenotype and reducing the migratory and invasive potential of pancreatic cancer cells. Taken together, the TRPM8 inhibitor AMTB suppresses EMT in pancreatic cancer. While the present study demonstrated that AMTB effectively inhibits EMT and downregulates MMP2/9 expression in pancreatic cancer cells, the precise molecular mechanisms were not fully dissected. Prior studies have extensively characterized the oncogenic role of TRPM8 and its downstream signaling pathways in pancreatic

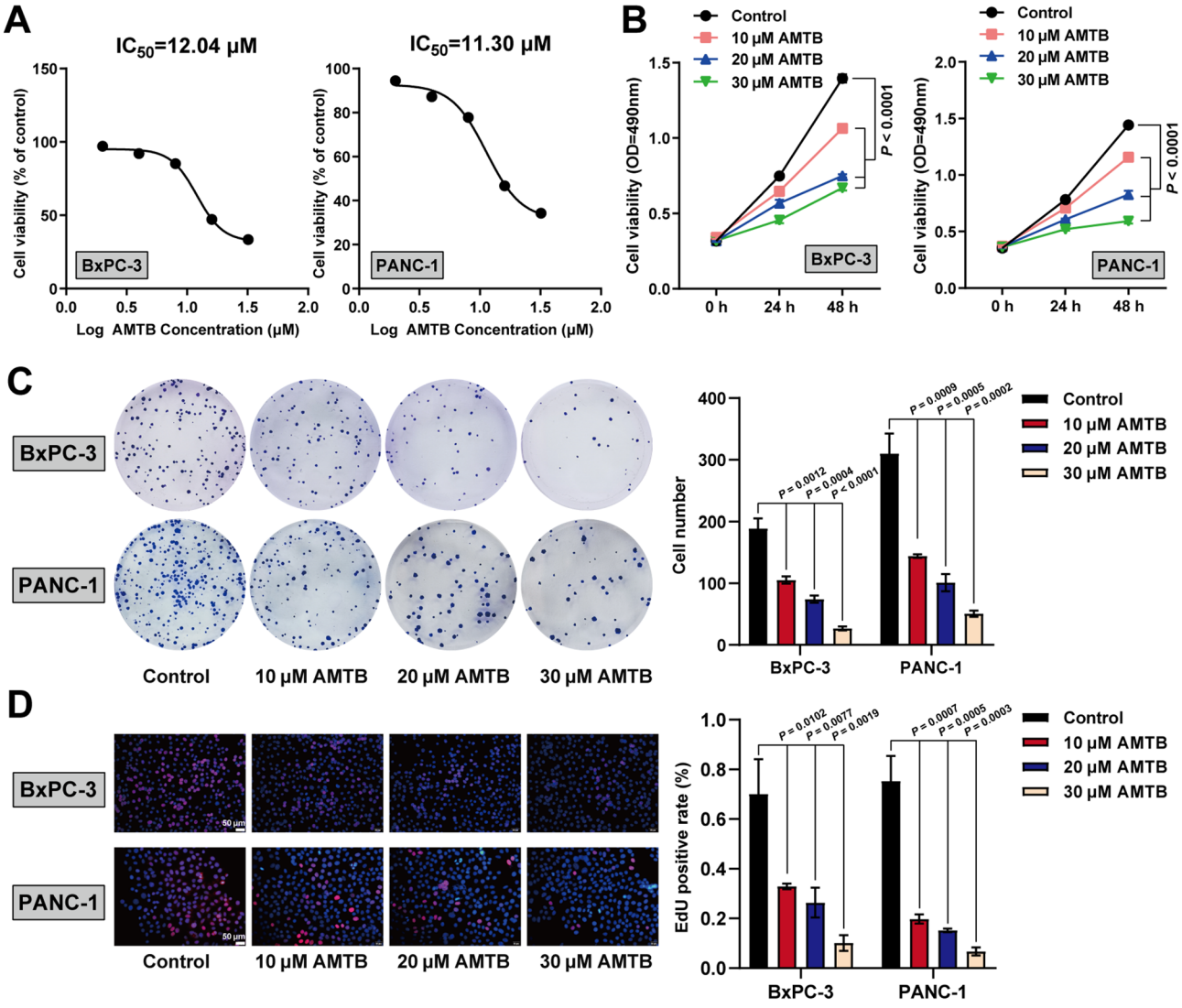

Fig. 1 TRPM8 antagonist AMTB inhibits pancreatic cancer cell proliferation. (**A**) Cell viability of BxPC-3 and PANC-1 treated with 0, 2, 4, 8, 16, or 32 μM AMTB was measured by MTT assay; IC50 values are shown. (**B**) Viability of AMTB-treated BxPC-3 and PANC-1 cells at various concentrations (10, 20, 30 μM) after 0, 24, and 48 h. C-D. Colony formation (**C**) and EdU staining (**D**) showed reduced proliferation in AMTB-treated cells. *N* = 3 (biological replicates)

and other cancers [39, 40], which laid the foundation for selecting TRPM8 as a therapeutic target in this study. To evaluate the therapeutic efficacy of a TRPM8-targeting strategy using a nanoparticle-based drug delivery system, AMTB was chosen over genetic approaches such as siRNA due to its superior chemical stability and reduced risk of off-target effects, which are common concerns in RNA-based therapeutics [41]. Future studies integrating TRPM8 knockdown or overexpression models would be valuable to further confirm the specificity of AMTB actions and to explore downstream signaling cascades in greater detail.

Herein, the role of AMTB in the modulation of MMP2/9 expression was revealed for the first time. MMP is a class of zinc endopeptidases exerting a crucial effect on the degradation of extracellular matrix (ECM) components and basement membrane (BM) [42]. MMP can be structurally separated into distinct subgroups, with MMP2 and MMP9 belonging to the gelatinase family. Within cancers, the excessive production or elevated activity of MMP2 and MMP9 can contribute to regulating apoptosis, proliferation, and angiogenesis through various mechanisms, including the promotion of ECM and BM degradation, thereby enabling the invasion of cancer cells into surrounding tissues and thereby achieving metastasis to distant organs [43]. A previous study has reported that TRPM8 agonists significantly upregulate the expression levels of MMP1, MMP3, and MMP13 [44]. The TRPM8 inhibitor AMTB in this study notably weakened the expression of MMP2/9, and these findings shed fresh light on the mechanism of AMTB's effect on pancreatic cancer cells as well as TRPM8's effect

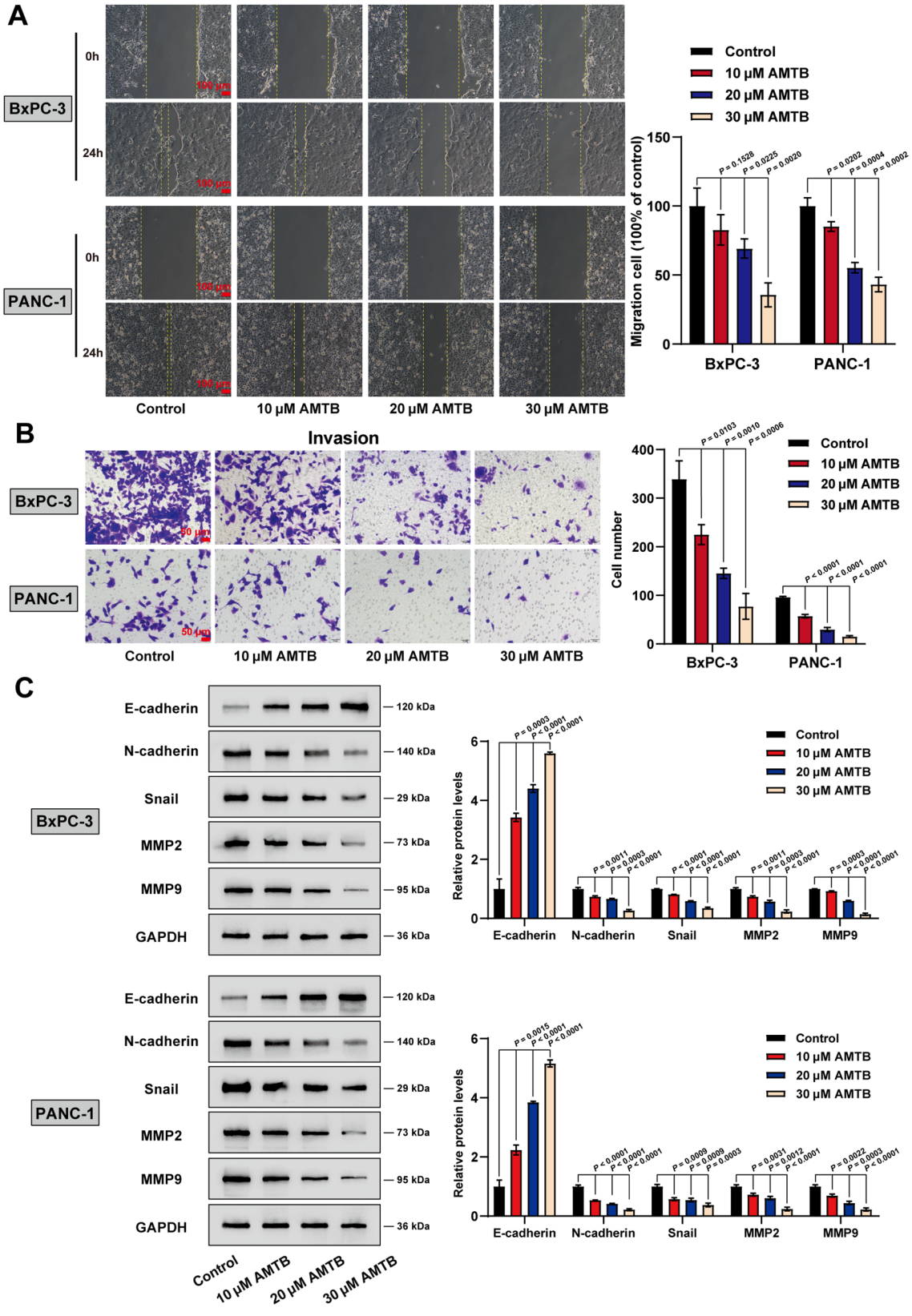

Fig. 2 TRPM8 antagonist AMTB restricts pancreatic cancer cell migration and invasion. (**A**) Scratch assay showing reduced migration in AMTB-treated BxPC-3 and PANC-1 cells. (**B**) Transwell assay indicating decreased invasion in AMTB-treated cells. (**C**) Western blot analysis of EMT-related proteins and migration/invasion markers (MMP2, MMP9) in AMTB-treated cells. $N = 3$ (biological replicates)

on MMP proteins. TRPM8 inhibition could suppress MMP2/9 expression through several potential mechanisms. One possible mechanism is the downregulation of transcription factors like Snail and Twist, which are known to regulate the expression of MMPs during EMT [45]. Additionally, TRPM8 antagonism could decrease the activation of the extracellular signal-regulated kinase (ERK) pathway, which is often linked to MMP expression and cancer cell invasion [46].

Characterization of CS-NPs@AMTB

The CS-NPs delivering the AMTB system were synthesized. The shape properties of CS-NPs and CS-NPs@AMTB were first characterized. Size is a key determinant of nanoparticle uptake, distribution, target accumulation and elimination in vivo [47]. Too large (> 200 nm) will be phagocytosed through the mononuclear phagocytosis system and accumulate within the liver and spleen, while too small (< 10 nm) will be cleared by the kidneys [48]. Herein, the average particle size of CS-NPs@AMTB increased to 124.4 nm compared to the 97.09 nm average particle size of CS-NPs (Fig. 3A). Both sizes meet the requirements of nanoparticles as drug delivery systems, and also indicate that AMTB has been successfully loaded onto CS-NPs. Meanwhile, the polydispersity index (PDI) of CS-NPs and CS-NPs@AMTB were 0.341 and 0.486. The zeta potentials of the NPs were further examined, and the zeta potentials of CS-NPs and CS-NPs@AMTB were 39.2 mV and 40.9 mV (Fig. 3B). CS is a positively charged polymer and the NPs generated in this study are consistent with previous studies [49] and drug loading did not significantly change the zeta potentials.

Shape is another critical fundamental characteristic of nanoparticles that influences and determines their in vivo bioprocesses. TEM images showed that CS-NPs exhibited a homogeneous and typical spherical shape, consistent with the shape of most currently available nanoparticles [48]. The morphology of CS-NPs did not change significantly after loading AMTB (Fig. 3C). This finding reveals that loading AMTB does not influence the basic structure and morphology of CS-NPs.

CS could lead to the controlled encapsulated drug release. AMTB was successfully loaded with 71.94% EE and 5.59% DL. The pH-dependent cumulative amount of AMTB released from the CS-NPs@AMTB is shown in Fig. 3D. The release of AMTB from CS-NPs was pH-dependent. AMTB were released dramatically faster under pH = 5.0 (representing the acidic environment found in tumors)than pH = 6.5 or pH 7.4 (mimicking normal physiological conditions), indicating that the drug release rates increased with decreasing pH due to the higher dissociation speed of the AMTB under the acidic pH environment. Moreover, AMTB exhibited sustained release behavior, and the cumulative AMTB release increased steadily over 12 h at pH 7.4, suggesting that AMTB was uniformly distributed in the formulation. Notably, the formulation showed a burst release within the first 4 h, which may be related to the faster release of the drug from small-sized NPs. Moreover, AMTB released up to more than 80% in the first 12 h, which is consistent with previous studies with CS-NP carriers [50]. The accelerated release at acidic pH conditions is consistent with the acidic tumor microenvironment, which may enhance targeted drug delivery to the tumor site. Moreover, Fig. 3E shows that the hemolysis rate of CS-NPs@AMTB with 160 μg/mL was 1.96%, which indicates slight hemolysis. The hemolysis rate of the other concentrations of CS-NPs and CS-NPs@AMTB was less than 3%. These results proved that both CS-NPs and CS-NPs@AMTB had excellent biocompatibility (Fig. 3E). Stability assessment in 10% FBS (pH 7.4) over 8 days demonstrated that particle size of CS-NPs@AMTB remained stable around 130 nm, indicating no significant aggregation or degradation of CS-NPs@AMTB under physiological conditions (Fig. 3F).

CS-NPs@AMTB diminishes pancreatic cancer cell proliferation and metastasis *in vitro*

Cellular uptake efficiency is important for nanoparticle-mediated tumor therapy. It was found that pancreatic carcinoma cell lines BxPC-3 and PANC-1 could effectively uptake CS-NPs and CS-NPs@AMTB (Fig. 4A). CS-NPs had no remarkable effect upon the biological behaviors of pancreatic cancer cell lines; whereas AMTB and CS-NPs@AMTB markedly weakened cell viability by about 40% and 50%, reduced proliferation by about 70% and 80%, inhibited migratory ability by about 40% and 60%, and reduced invasive ability about 50% and 80% of pancreatic cancer cells (all $P < 0.01$, Fig. 4B-E). Moreover, the effects of CS-NPs@AMTB on cell viability, proliferation, migratory ability, and invasive ability of non-cancerous pancreatic ductal epithelial cells (HPDE6-C7) were also investigated. As the results of supplementary Fig S2 show, CS-NPs@AMTB had no significant effect on the cell viability (Fig S2A), cell proliferation (Fig S2B), cell migration (Fig S2C) and invasion (Fig S2D) of HPDE6-C7 cells (all $P > 0.05$). Next, CS-NPs had no significant impact on the expression levels of EMT-associated proteins and MMP2/9; while AMTB and CS-NPs@AMTB significantly increased E-cadherin levels by about 1.5-fold and 2-fold, and reduced the expression levels of cellular EMT-related proteins (N-cadherin and Snail) and MMP2/9 by about 40% and 70% (all $P < 0.05$, Fig. 4F). Notably, the use of CS-NPs@AMTB was associated with better tumor cell

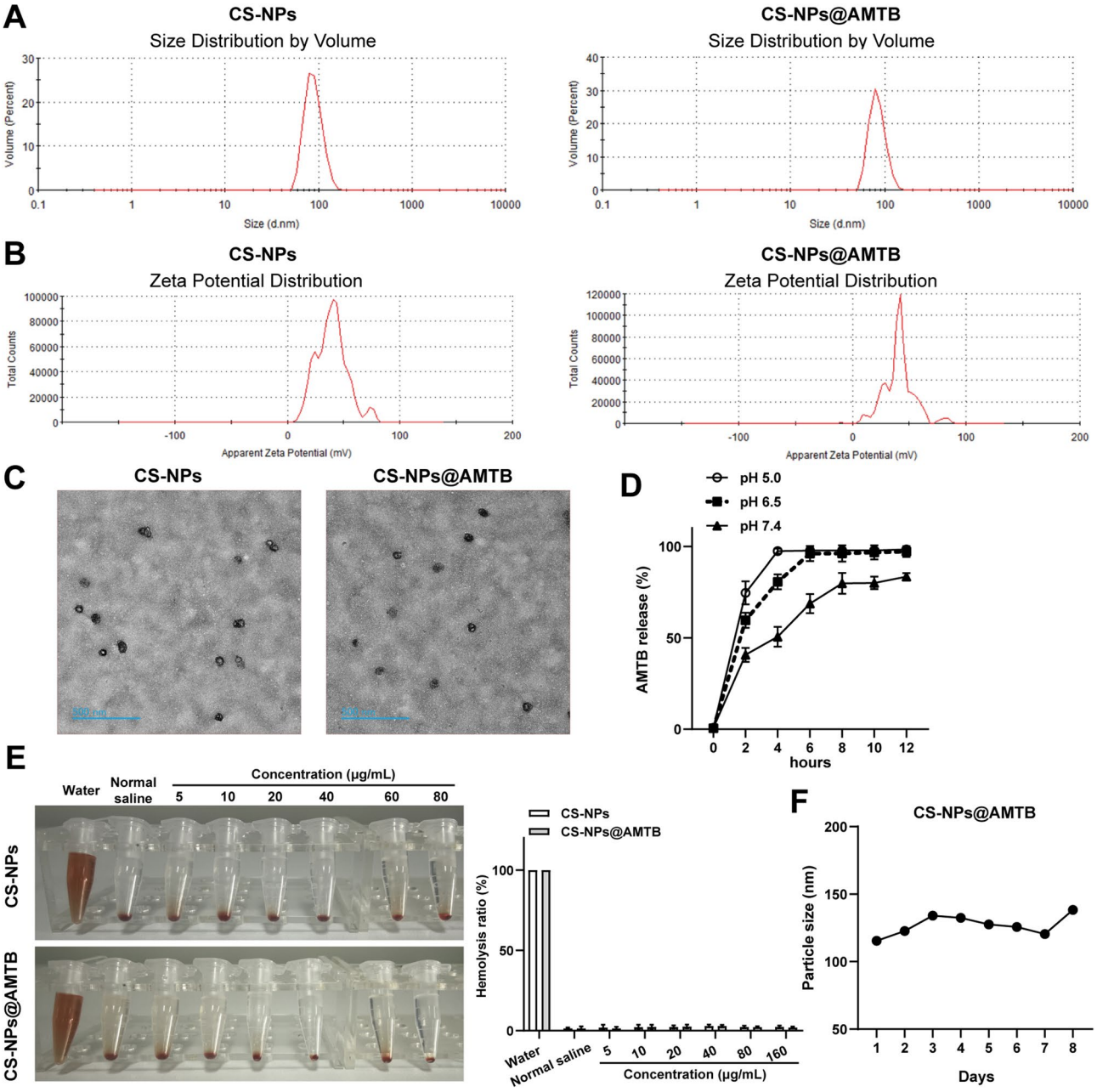

Fig. 3 Characterization of CS-NPs@AMTB. (**A**). The average particle size of CS-NPs and CS-NPs@AMTB was assessed using dynamic light scattering (DLS). (**B**): Measurement of the zeta potentials of CS-NPs and CS-NPs@AMTB. (**C**). The surface morphology of CS-NPs and CS-NPs@AMTB was measured using transmission electron microscopy (TEM). (**D**). The release pattern of AMTB from CS-NPs@AMTB at pH 7.4, 6.5 and 5.4. (**E**) Hemolysis ratios of erythrocytes treated with different concentrations of CS-NPs and CS-NPs@AMTB. (**F**) Stability assessment of CS-NPs@AMTB nanoparticles showing particle size variation over 8 days in 10% fetal bovine serum (FBS, pH 7.4)

killing and inhibition of EMT and MMP2/9 compared to free AMTB alone (all $P < 0.05$, Fig. 4B-F). This enhanced therapeutic effect can be attributed to the specific accumulation of CS-NPs@AMTB in tumor cells, resulting in improved anti-tumor activity against pancreatic cancer cells. These findings highlight the promise of CS-NPs@AMTB for targeted drug delivery and cancer therapy.

CS-NPs@AMTB diminishes the proliferation and metastasis of pancreatic cancer cells *in vivo*

Consistent with the findings of the in vitro experiments, CS-NPs had no significant influence on the malignant phenotype of pancreatic cancer cells, whereas AMTB and CS-NPs@AMTB were able to notably suppress pancreatic cancer cell tumorigenicity by about 40% and 70% (all $P < 0.05$, Fig. 5A-D) and tumor proliferation (Fig. 5E) and

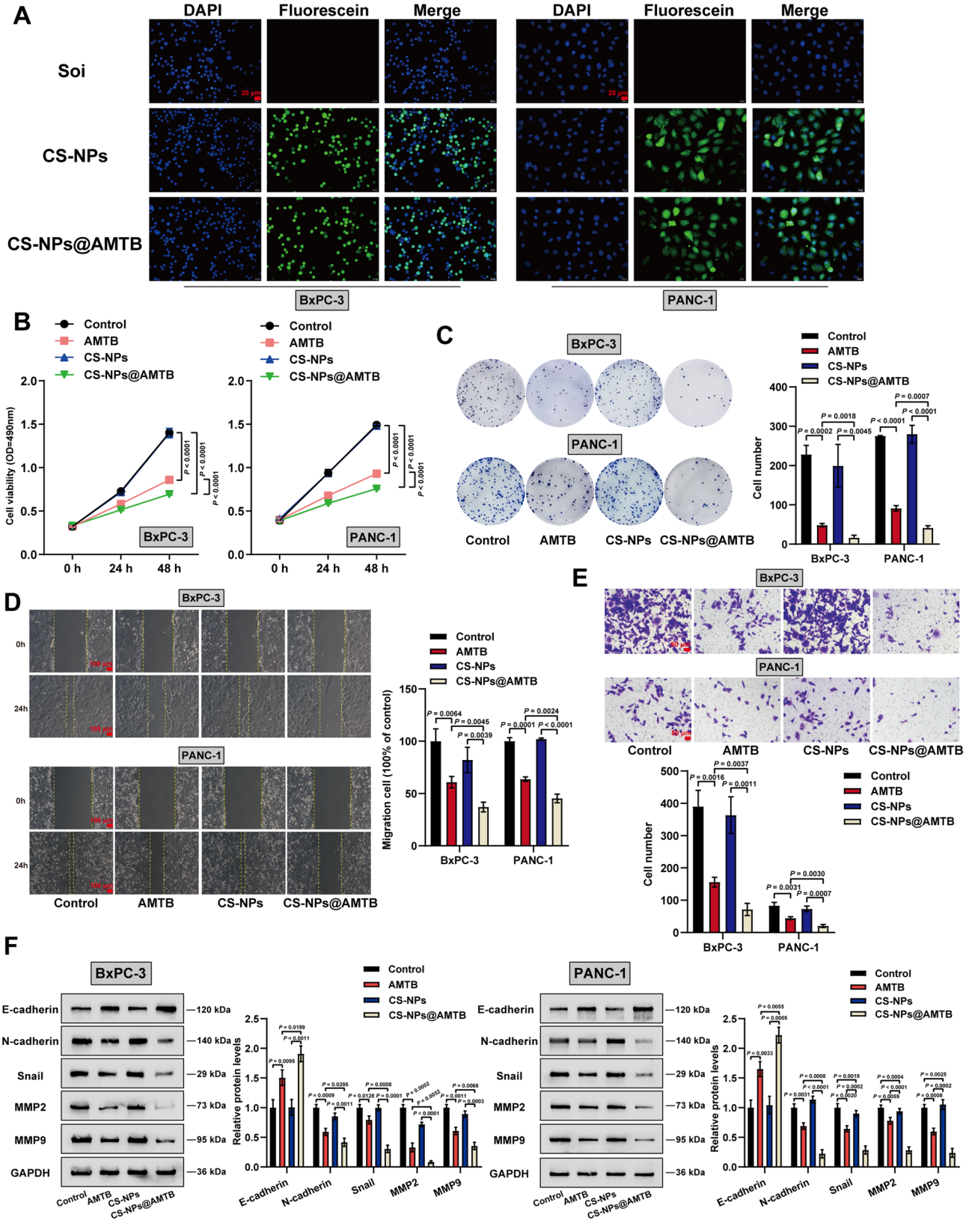

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Fig. 4 CS-NPs@AMTB diminishes the proliferation and metastasis of pancreatic cancer cells *in vitro*. (**A**). CS-NPs and CS-NPs@AMTB were labeled separately using Soi to detect the uptake efficiency of pancreatic cancer cells on nanomaterials. BxPC-3 and PANC-1 cells were treated with 20 μM AMTB or 151 μg/mL of CS-NPs, or 160 μg/mL of CS-NPs@AMTB for 24 h, and then were divided into four group: control group, AMTB group, CS-NPs and CS-NPs@AMTB. (**B**-**E**). Cell viability, proliferation, migration, and invasion assays following treatment with AMTB, CS-NPs, or CS-NPs@AMTB using MTT (**B**), colony formation (**C**), scratch (**D**) and Transwell (**E**) assays, respectively. (**F**). Western blot analysis of EMT markers and MMP2/9 in treated cells. $N = 3$ (biological replicates)

promoted tumor apoptosis (Fig. 5F) in nude mice. Moreover, AMTB and CS-NPs@AMTB inhibited the number of lung nodules by about 20% and 60% and reduced lung weight by about 26% and 60% in the mouse pancreatic cancer lung metastasis model (all $P < 0.01$, Fig. 5G-I), as well as suppressed N-cadherin, snail, and MMP2/9 expression levels by about 30% and 70%, and promoted E-cadherin levels by about 2-fold and 2.5-fold in lung metastatic tumor tissues (all $P < 0.05$, Fig. 5J). Compared to free AMTB, CS-NPs@AMTB exhibited better therapeutic effects (all $P < 0.05$, Fig. 5A-J). This enhanced tumor suppression can be attributed to the improved bioavailability and sustained release of AMTB within the tumor microenvironment. The encapsulation of AMTB in chitosan nanoparticles not only protects the drug from rapid degradation but also facilitates better retention at the tumor site, leading to more effective tumor inhibition. Numerous studies have explored the use of various nanoparticles, such as liposomal formulations [51], polymeric nanoparticles [52], and gold nanoparticles [53], for targeted drug delivery in pancreatic cancer. Our findings with CS-NPs@AMTB are consistent with this growing body of research, demonstrating significant anti-cancer effects. However, one distinguishing feature of CS-NPs@AMTB is its ability to directly target and modulate the TRPM8 channel, a promising target in pancreatic cancer. The synergistic effects of CS-NPs@AMTB with other treatment modalities, such as chemotherapy (e.g., gemcitabine) or immunotherapy, warrant further investigation. In particular, combining nanoparticle-based therapies with immune checkpoint inhibitors or PI3K-Akt pathway inhibitors may enhance therapeutic efficacy by modulating the tumor microenvironment and enhancing the anti-cancer immune response [54]. Although the present study was designed primarily to investigate the therapeutic efficacy of TRPM8 inhibition in pancreatic cancer, it did not include a direct comparison with standard chemotherapeutic agents such as gemcitabine. Given the clinical relevance of gemcitabine-based regimens, future studies are warranted to evaluate the comparative efficacy and potential synergistic effects of CS-NPs@AMTB in combination with conventional chemotherapeutics, which may further improve therapeutic outcomes in pancreatic cancer.

The effects of CS-NPs@AMTB on the heart, liver, spleen, and kidney in healthy mice were examined to assess the biosafety of the nanoparticles. HE staining results demonstrated that there was no significant tissue damage or abnormalities in the cellular structure in the major organ tissues of AMTB, CS-NPs, and CS-NPs@AMTB-treated mice (Fig. 5K), suggesting that the formulation offers a safe and effective means of delivering AMTB to the tumor site. Chitosan is mainly derived from seafood by-products and the thereof CS-NPs are known as nontoxic, biocompatible, biodegradable and functionalized nanostructures [55]. Numerous studies have indicated that neither in vitro cell culture experiments nor animal models have shown significant cellular toxicity or tissue damage from CS-NPs [56]. These findings revealed the safety and feasibility of CS-NPs@AMTB as a potential anti-pancreatic cancer therapeutic modality.

TRPM8 expression is increased in pancreatic cancer and is associated with patients' unfavorable prognosis

Pancreatic cancer and adjacent non-cancerous tissues were collected from 15 pairs of pancreatic carcinoma patients, and histopathological changes were detected using HE staining (Fig. 6A). IHC staining, qRT-PCR, and western blot were performed to determine TRPM8 expression within the tissue samples, and the results revealed that TRPM8 expression was remarkably elevated within pancreatic carcinoma tissue samples relative to non-cancerous tissue samples by about 1.5-fold (all $P < 0.01$, Fig. 6B-D). Moreover, the correlation between TRPM8 expression and clinicopathological features of pancreatic cancer patients is listed in Table S1. There was no significant association between TRPM8 level and age or gender, while patients with high expression of TRPM8 were more prone to reach an advanced TNM stage, lymph node metastasis, and perineural invasion ($P < 0.05$). TRPM8 mRNA expression and protein level were markedly elevated by about 2-fold, 2-fold, 1.5-fold, 1.5-fold within four pancreatic carcinoma cell lines (BxPC-3, PANC-1, PATU-8988, and AsPC-1) compared with non-cancerous pancreatic ductal epithelial cells HPDE6-C7 (all $P < 0.05$, Fig. 6E-F). These results further validate that the high TRPM8 expression within pancreatic carcinoma is consistent with previous studies [57]. A previous study has shown that abnormally high TRPM8 expression within colorectal carcinoma could predict patients' unfavorable prognosis [8]. Here, TRPM8 expression was analyzed using the Kaplan-Meier Plotter online tool, which integrates data from multiple public datasets including TCGA, GEO, and EGA [58]. Kaplan-Meier survival analysis based on TCGA-PAAD and pancreatic cancer gene chips revealed that patients with high TRPM8

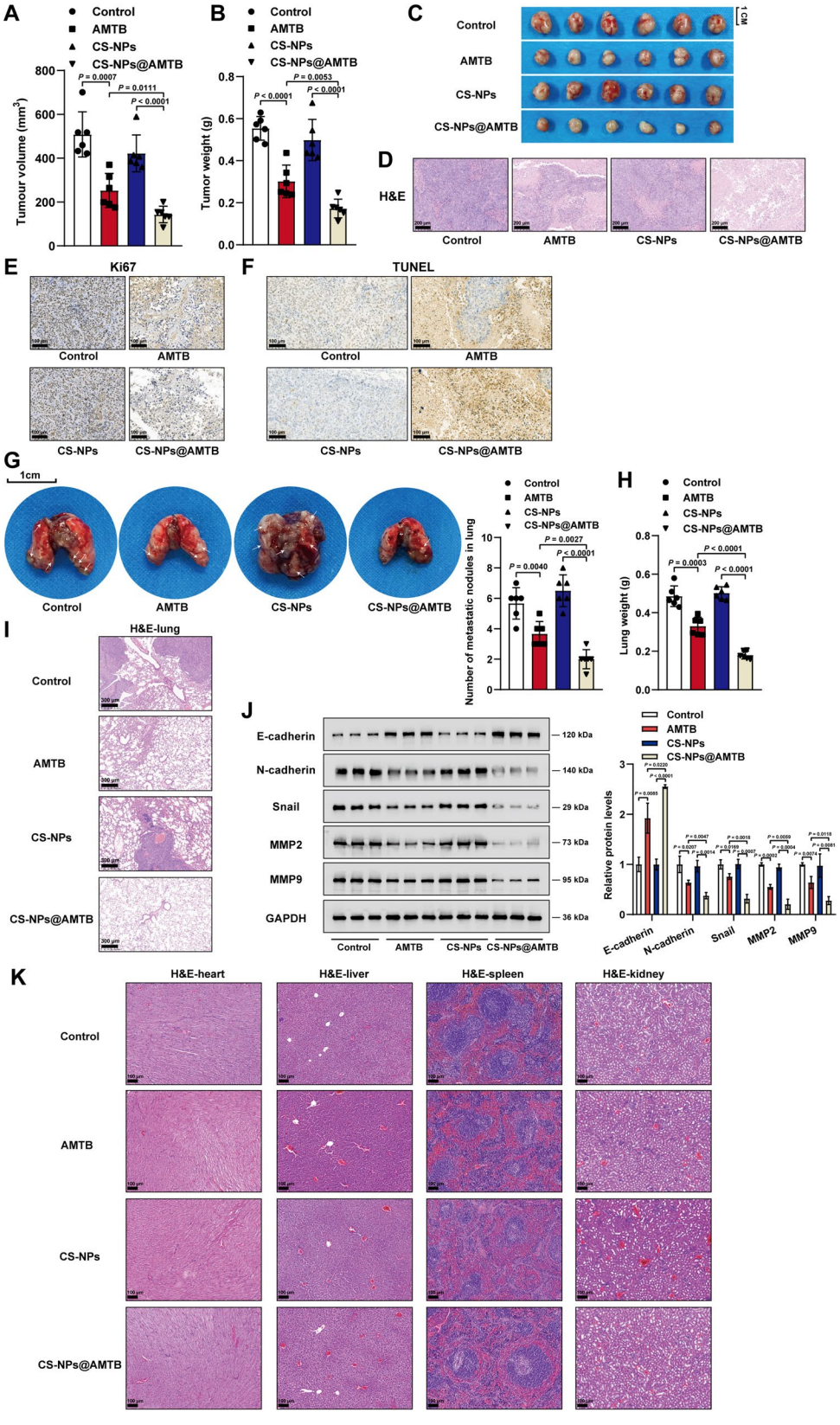

Fig. 5 (See legend on next page.)

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Fig. 5 CS-NPs@AMTB diminishes pancreatic cancer cell proliferation and metastasis *in vivo*. BALB/c nude mice were induced into the tumor model by subcutaneous injection of PANC-1 cells, and after the subcutaneous tumor volume reached 100 mm^3, the mice were treated using intraperitoneal injections of AMTB, CS-NPs, and CS-NPs@AMTB, respectively, which were administered every 2 days, and the mice were euthanized at day 25. (**A**-**B**). Assessment of tumor volume (**A**) and weight (**B**) ($n=6$). (**C**). Gross structure of tumor tissues in each group of mice ($n=6$). (**D**-**F**). HE staining (**E**), IHC staining for Ki67 (**E**) and TUNEL staining (**F**) were conducted to characterize pathological changes, tumor proliferation, and tumor apoptisis of cancerous tissues ($n=3$). C57BL/6 mice were injected by tail vein with the mouse pancreatic cancer cell line Panc02 cells to induce a lung metastatic tumor model. Intraperitoneal injection of AMTB, CS-NPs, and CS-NPs@AMTB was carried out on day 9 of the experiment at two-day intervals, for a total of five doses, and the mice were euthanized at day 21 of the administration, and the pulmonary tissues were collected for the subsequent experiments. (**G**). Gross structural and quantitative statistics of metastases in pulmonary tissues of mice from all groups. (**H**). Weight of lung tissues of mice from all groups. (**I**). HE staining was carried out to observe pathological alterations within pulmonary tissues. (**J**). The expression levels of EMT-associated proteins as well as MMP2 and MMP9 in lung metastatic tumor tissues were detected using Western blot. (**K**). In vivo safety evaluation: healthy C57BL/6 mice were injected intraperitoneally with AMTB, CS-NPs, and CS-NPs@AMTB every two days for five doses, and the mice were sacrificed on the 12nd day of administration to harvest the hearts, livers, spleens, and kidneys. The pathological alterations were detected using HE staining. $N=6$ (biological replicates)

expression had significantly shorter overall survival compared to those with low expression levels ($P<0.01$, Fig. 6G-H). These findings further support the important effect of TRPM8 on pancreatic cancer and suggest that it might serve as a potential prognostic indicator.

The limitations of this study should also be noted. Firstly, this study relied on mouse models and cell lines, which may not fully capture the complexity of human pancreatic cancer. As a results, the findings observed in these preclinical models may not be entirely translatable to human patients, and further studies using human-derived models are necessary to improve the generalizability of our results. Secondly, this study only investigated the effect of one TRPM8 inhibitor (AMTB) on pancreatic cancer. The effects of other TRPM8 inhibitors, such as RQ-00203078 [22], on pancreatic cancer are unclear. The role of these alternative TRPM8 antagonists in pancreatic cancer should be explored in future research to assess their therapeutic efficacy and potential advantages over AMTB. Thirdly, due to pancreatic cancer's early metastasis and limited response to the current therapies, nanoparticle therapy alone may not be effective against pancreatic cancer. Therefore, in future experiments, nanotherapy could be combined with other pancreatic cancer treatments, such as chemotherapy (e.g., gemcitabine) or immunotherapy, to explore their joint effects on pancreatic cancer. Furthermore, while the use of CS-NPs@AMTB shows significant therapeutic potential, scaling up nanoparticle production for clinical applications presents several challenges that must be addressed for successful translation into the clinic. One of the primary challenges is ensuring consistent and reproducible nanoparticle characteristics during large-scale production. The stability of nanoparticles in biological fluids is another consideration. Moreover, scalability and manufacturing costs must be addressed to make nanoparticle-based therapies economically viable for widespread clinical use. Additionally, the production process needs to be further optimized to meet the standards required for regulatory approval. Future research should focus on further optimizing nanoparticle formulations to improve drug loading efficiency, release rates, and stability in biological environments. Additionally, clinical validation through pharmacokinetic studies and human-derived models, such as patient-derived xenografts (PDX) or organoid models, is critical to assess the safety, efficacy, and therapeutic potential of CS-NPs@AMTB in a clinical setting.

Although TRPM8 is predominantly studied in the context of tumor progression, it also plays physiological roles in non-cancerous tissues, particularly in thermosensation and pain signaling pathways [59, 60]. While no behavioral abnormalities or systemic toxicity were observed in vivo at the administered doses, and no cytotoxic effects were detected in normal pancreatic ductal epithelial cells, the possibility of off-target effects cannot be fully excluded. The use of CS-NPs might contribute to preferential tumor accumulation and reduced systemic exposure; however, future pharmacological and toxicological evaluations are warranted to comprehensively assess the safety profile of TRPM8 inhibition in non-tumor contexts. In addition, although the current in vivo experiments were limited to short-term assessments, the sustained tumor-suppressive and anti-metastatic effects observed suggest that CS-NPs@AMTB might exert durable therapeutic benefits. Previous studies have demonstrated that chitosan-based nanoparticles possess favorable pharmacokinetic properties, including prolonged circulation time and enhanced tumor retention [61, 62]. These features, together with the robust in vivo efficacy shown here, support the potential of CS-NPs@AMTB for long-term tumor control. Nonetheless, future studies incorporating extended observation periods, recurrence monitoring, and survival analysis are warranted to fully evaluate the long-term therapeutic impact. Also, although this study did not include pharmacokinetic or pharmacodynamic (PK/PD) evaluations, the observed in vivo anti-tumor efficacy and absence of major systemic toxicity indirectly suggest effective biodistribution and tumor accumulation of CS-NPs@AMTB. Nevertheless, comprehensive PK/PD analyses, such as quantification of drug concentrations in plasma and tumor tissues over time, are essential for elucidating nanoparticle clearance, systemic exposure, and tumor-targeting kinetics. Future studies incorporating

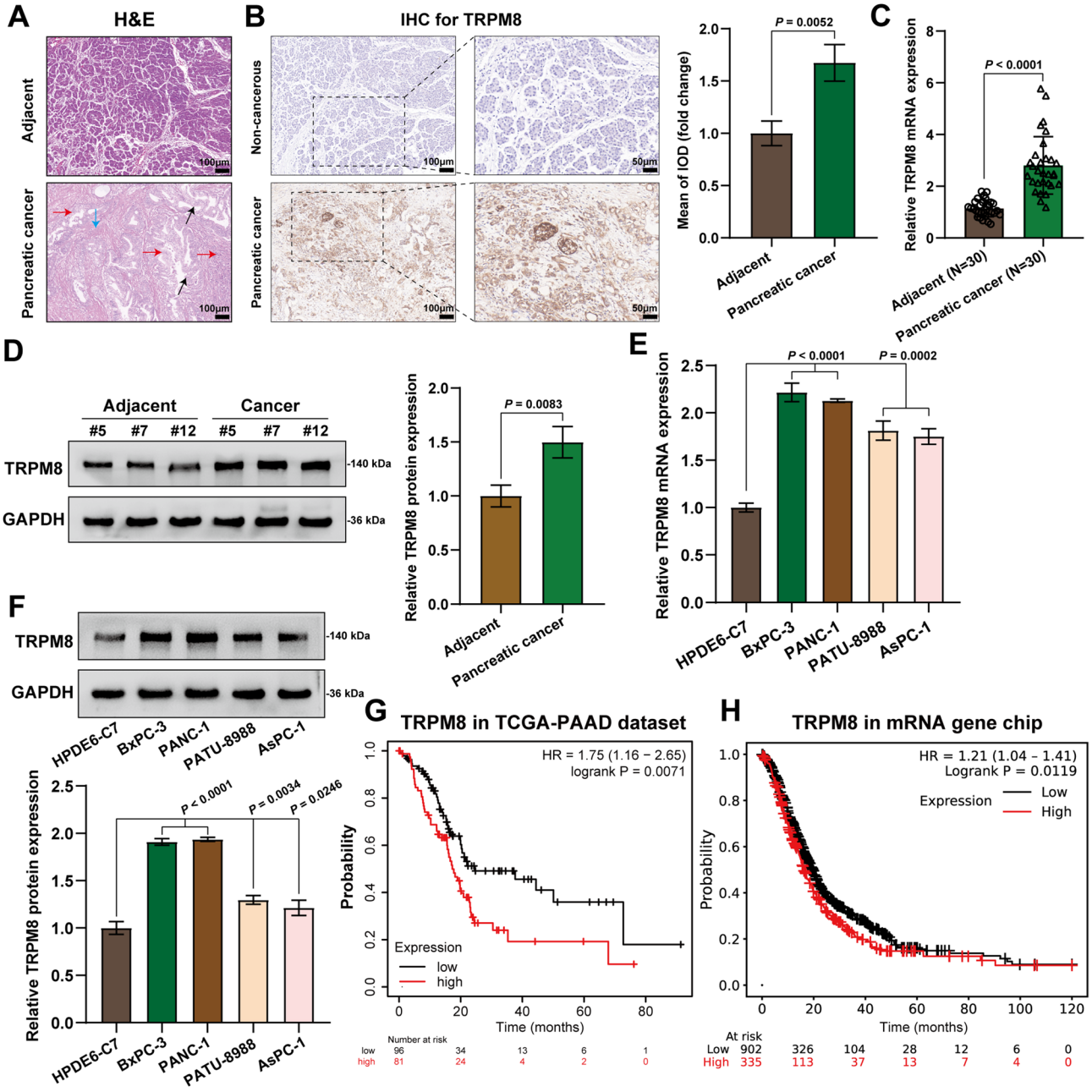

Fig. 6 TRPM8 expression is elevated in pancreatic cancer and correlates with patients' poor prognosis. (**A**). The pathological changes in cancerous and adjacent non-cancerous tissues were identified using HE staining. Black arrow indicates the irregular tubular structure; red arrow indicates columnar epithelium cells; blue arrow indicates the fibrous connective tissue between ducts. (**B**-**D**). The elevated TRPM8 protein and mRNA expression levels were detected by IHC staining (**B**), qRT-PCR (**C**) and western blot (**D**) in cancerous tissue samples compared to adjacent non-cancerous tissue samples. (**E**-**F**). High TRPM8 mRNA and protein expression were observed in pancreatic carcinoma cell lines (BxPC-3, PANC-1, PATU-8988, and AsPC-1) compared to non-cancerous pancreatic ductal epithelial cells (HPDE6-C7), as detected by qRT-PCR (**E**) and western blot (**F**). (**G**-**H**) Kaplan-Meier analysis of the correlation between TRPM8 expression and patient prognosis based on online tool Kaplan-Meier Plotter (https://kmplot.com/analysis/). Kaplan-Meier survival analysis of TRPM8 expression in TCGA-PAAD (**G**) and gene chip (**H**). Patients with high TRPM8 expression (red line) exhibited significantly poorer overall survival compared to those with low expression (black line) (HR = 1.75, 95% CI = 1.16–2.65, Logrank *P* = 0.0071; HR = 1.21, 95% CI = 1.04–1.41, Logrank *P* = 0.0119)

such assessments will be crucial for supporting the clinical translation and optimization of this delivery platform. Given the highly aggressive and treatment-resistant nature of pancreatic cancer, future studies should explore the therapeutic potential of combining CS-NPs@AMTB with standard-of-care regimens such as gemcitabine or FOLFIRINOX. Additionally, as TRPM8 has been implicated in modulating tumor immunity, integrating CS-NPs@AMTB with immune checkpoint inhibitors might offer synergistic benefits by enhancing both direct tumor

suppression and immune-mediated responses. Such combination strategies might broaden the clinical applicability of CS-NPs@AMTB and improve treatment efficacy in advanced or refractory pancreatic cancer.

Conclusion

In this study, we developed and evaluated, for the first time, a CS-NPs system encapsulating the TRPM8 inhibitor AMTB for the treatment of pancreatic cancer. The CS-NPs@AMTB formulation was effectively internalized by pancreatic cancer cells and exhibited potent anti-proliferative and anti-metastatic effects both in vitro and in vivo. These findings support CS-NPs@AMTB as a promising candidate for targeting both primary and metastatic pancreatic cancer. Notably, CS-NPs@AMTB demonstrated superior efficacy compared to free AMTB, highlighting the potential advantages of nanoparticle-based delivery in enhancing drug stability, bioavailability, and tumor-targeting capacity, while addressing challenges such as poor solubility and systemic off-target effects.

While the present study did not include direct comparisons with standard chemotherapeutics, the favorable outcomes observed here provide a strong rationale for further investigation into combinatorial strategies, particularly with agents such as gemcitabine. To advance CS-NPs@AMTB toward clinical application, future studies should include assessments of long-term efficacy, pharmacokinetics, and safety in large animal models. In parallel, efforts to optimize formulation stability, large-scale production processes, and regulatory compliance will be necessary to support clinical development. Ultimately, well-designed clinical trials will be essential to evaluate the therapeutic potential of CS-NPs@AMTB, either as monotherapy or in combination with existing treatments, for the personalized management of pancreatic cancer.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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None.

Author contributions
Jiefeng Liu and Yong Gao contributed to the experimental design and supervising the whole experimental process; Yujing Gong, Xinyu Zeng, and Miao He were involved in the experimental conducting; Bin He and Wenbin Gao contributed to the data analysis and manuscript preparation; Jiefeng Liu collected grants. All the authors read, revised, and approved the final manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the Fourth Hospital of Changsha and with the 1964 Helsinki Declaration. Informed consent to participate in the study has been obtained from participants. The guidelines for the care and use of animals were approved by the Medicine Animal Welfare Committee of the Fourth Hospital of Changsha.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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