Glypican-1-targeted and gemcitabine-loaded liposomes enhance tumor-suppressing effect on pancreatic cancer

Yu Mu¹, Dezhi Wang², Liangyu Bie¹, Suxia Luo¹, Xiaoqian Mu¹, Yanqiu Zhao¹

¹Department of Oncology, Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, Henan Province, China
²East China Normal University, Shanghai, China

Correspondence to: Yanqiu Zhao; email: 16530907585@163.com; https://orcid.org/0000-0003-1106-8097
Keywords: liposome, phosphatidylinositol proteosan-1, gemcitabine, orthotopic pancreatic cancer mice, pancreatic cancer
Received: February 12, 2020  Accepted: July 25, 2020  Published: October 9, 2020

ABSTRACT

Liposomes (LPs) as promising drug delivery systems are widely applied in cancer therapy. This study aimed to investigate the effect of glypican-1 (GPC1)-targeted and gemcitabine (GEM)-loaded LP [GPC1-LP (GEM)] on cell proliferation and apoptosis in PANC-1s, as well as on orthotopic pancreatic cancer (PDAC) mice. The GPC1-LP (GEM) and LP (GEM) was prepared, and then the size distribution of GPC1-LP (GEM) was analyzed by dynamic light scattering (DLS). In vitro drug release assay of GPC1-LP (GEM) and LP (GEM) was performed, and the expression of GPC1 in PANC1 cells was detected as well. Next, the effects of free GEM, LP (GEM) and GPC1-LP (GEM) on cell viability, clone number, and apoptosis, as well as the expression of proteins associated with apoptosis were measured in 239T and PANC-1 cells. Furthermore, the body weight and tumor size of orthotopic PDAC mice were evaluated following the treatment of free GEM, LP (GEM) or GPC1-LP (GEM). LP (GEM) and GPC1-LP (GEM) were successfully prepared with a successful GEM release within 24 h. In addition, GPC1 was positively expressed in PANC-1 cells but not 293T cells. These findings provided more insights into the anti-tumor potential for the biomedical application of GPC1-LP (GEM) in PDAC.

INTRODUCTION

As one of the common alimentary canal malignant tumors, pancreatic cancer (PDAC) usually originates from the ductal epithelium, with high rates of morbidity and mortality [1]. The 5-year survival rate of PDAC is about 6%, thus, PDAC is considered as one of the worst malignant tumors [2]. Nowadays, the conventional and effective therapeutic methods for PDAC include targeted pharmacological treatments, sophisticated surgical resection and advanced chemotherapy [3–5]. Despite the intensive progress in diagnosis and treatments, poor survival and unsatisfactory prognosis remain an issue due to delayed diagnosis and high metastasis [2]. Therefore, it is essential to search for new drugs and novel treatment methods for PDAC.

It is well known that Gemcitabine (GEM), a cytosine derivative, is used as the first-line chemotherapeutics for unresectable PDAC [6]. Previous studies have demonstrated that GEM combined with other drugs can improve overall survival and progression-free survival of PDAC patients [7–9]. However, the clinical use of GEM is compromised for efficient cancer treatment due to its short half-life, low bioavailability, and other side effects caused by nonspecific cytotoxicity [10]. Thus, it is indispensable to design an effective targeted drug delivery system that are able to increase the concentration of drugs in tumor cells and reduce the distribution of drugs in normal tissues and organs, thereby reducing toxic side effects.

Currently, liposomes (LPs) are consisted of phospholipids, which have been reported to be a promising drug delivery system, and widely applied in cancer therapy [11]. As the most common nanocarriers, LPs possess several favorable properties such as high biocompatibility, ability to carry large drug payloads,
and capacity for self-assembly [12]. Based on these advantages, LPs are conducive to improve biodistribution of compounds to target sites, overcome obstacles to cellular and tissue uptake, and stabilize therapeutic compounds in vivo [12]. Importantly, previous studies have developed GEM-loaded LPs [LP (GEM)], and LP (GEM) exhibits improved tumor-suppressing effects compared with free GEM by improving the chemotherapy resistance and decreasing the systemic toxicity [13, 14].

Notably, glypican-1 (GPC1) is a proteoglycan anchored to the surface of cell membranes, which is highly expressed in the lesion tissues of PDAC, but not expressed or under-expressed in normal tissues [15]. Therefore, we speculated that GPC1-targeted LP (GEM) [GPC1-LP (GEM)] might further improve tumor-suppressing effects due to more accurate drug targeting. In the current research, GPC1-LP (GEM) was successfully prepared, and then characterization and in vitro drug release of GPC1-LP (GEM) were detected. In addition, the effects of GPC1-LP (GEM) on cell proliferation and apoptosis in PANC-1s, as well as on orthotopic PDAC mice were explored.

RESULTS

Characterization and in vitro drug release of GPC1-LP (GEM)

As shown in Figure 1A, DLS revealed that the hydrodynamic diameter of GPC1-LP (GEM) was observed to be around 100 nm, which suggested the successful establishment of GPC1-LP (GEM). Next, the amount of GEM released from LP (GEM) and GPC1-LP (GEM) was examined at pH 7.4 and pH 5.0. Cumulative drug release profiles revealed that both LP (GEM) and GPC1-LP (GEM) exhibited a burst release of GEM within 5 h and a slow release from 5 h to 24 h at pH 7.4 (blood plasma) and pH 5.0 (tumor endocytic compartment) (Figure 1B). Notably, almost 80% of GEM was released from both LP (GEM) and GPC1-LP (GEM) within 24 h at pH 5.0, which was significantly higher than that at pH 7.4 (30%) (Figure 1B), indicating more GEM releasing into the tumor environment.

Expression of GPC1 in PANC-1 cells

GPC1 was highly expressed in pancreatic cancer, as predicted by GEPIA, a tumor-related database (Figure 2A). The expression levels of GPC1 were measured by qRT-PCR and western blotting showed higher expression level of GPC1 in PANC-1 and SW1990 cells than in 293T cells (Figure 2B, 2C). Consistently, cell immunofluorescence showed the positive expression of GPC1 in PANC-1 but not 293T cells (Figure 2D). These results suggested that PANC-1 and SW1990 cells were GPC1-overexpressed cells.

In vitro anti-proliferation effect of GPC1-LP (GEM)

Cell proliferation and apoptosis were used to evaluate the anti-tumor effect of GPC1-LP (GEM). MTT assay showed that 293T cells treated with LP (GEM) exhibited similar cell viability with 293T cells treated with GPC1-LP (GEM) at 24 h and 48 h, while both were reduced compared with cells treated with free GEM (Figure 3A). Compared with PANC-1 cells with free GEM, cell viability was decreased in PANC-1 cells treated with LP (GEM), and cells treated with GPC1-LP (GEM) showed lower cell viability than cells treated with LP (GEM) at 24 h and 48 h (Figure 3A). Consistently, colony formation results also revealed that compared with control cells, the clone number was decreased in 293T cells treated with free GEM (Figure 3B).
number was similar between 293T cells treated with LP (GEM) and GPC1-LP (GEM), while which were decreased compared to 293T cells treated with free GEM (P < 0.05, Figure 3B). In PANC-1 and SW1990 cells, the clone number in cells treated with GPC1-LP (GEM) was the lowest, followed by cells treated with LP (GEM), cells treated with LP (GEM), cells treated with free GEM, and control cells (P < 0.05, Figure 3B). These data indicated that GPC1-LP (GEM) had superior anti-proliferation effect than LP (GEM) and free GEM in PANC-1 and SW1990 cells. (Figure 3).

In vitro pro-apoptosis effect of GPC1-LP (GEM)

Flow cytometry analysis found similar cell apoptosis in control cells, cells treated with LP, and cells treated with GPC1-LP, while the rate of apoptotic cells was increased in cells with free GEM compared with control cells, cells with LP, and cells with GPC1-LP; meanwhile, compared with cells with free GEM, cells with LP (GEM) showed the increased rate of apoptotic cells, and then GPC1-LP (GEM) treatment further increased the rate of apoptotic cells (Figure 4A).

Figure 2. Expression of GPC1 in 293T cells, SW1990 cells and PANC-1 cells. (A) GPC1 expression in pancreatic cancer was predicted by GEPIA. The mRNA and protein levels of GPC1 in 293T cells, SW1990 and PANC-1 cells by (B) qRT-PCR and (C) western blotting. (D) The expression of GPC1 in 293T cells and PANC-1 cells by cell immunofluorescence. bar = 20 μm. *p<0.05, ***p<0.001.
Consistently, western blotting showed that compared with control cells, free GEM treatment distinctly inhibited Bcl-2 level as well as promoted the expression of Bax, cleaved-caspase-3 and cleaved-caspase-9 ($P < 0.01$), and both LP (GEM) and GPC1-LP (GEM) further inhibited Bcl-2 level as well as promoted the expression of Bax cleaved-caspase-3 and cleaved-caspase-9, especially GPC1-LP (GEM) ($P < 0.05$, Figure 4B).

These findings suggested that superior pro-apoptosis effect of GPC1-LP (GEM) than LP (GEM) and free GEM in PANC-1 and SW1990 cells.

**In vivo therapeutic effect of GPC1-LP (GEM)**

*In vivo* experiments showed that the bodyweight of mice with different treatments, including LP, GPC1-LP,

---

**Figure 3.** GPC1-LP (GEM) inhibited cell growth in 293T cells, PANC-1 cells and SW1990 cells. (A) Cell viability of 293T cells, PANC-1 cells and SW1990 cells treated with different doses of GEM, LP (GEM) and GPC1-LP (GEM) at 24h and 48 h by MTT assay. (B) Clone number of 293T cells, PANC-1 cells and SW1990 cells treated with PBS (control), GEM, LP (GEM) and GPC1-LP (GEM) by colony formation assay. *p<0.05.
Figure 4. GPC1-LP (GEM) induced cell apoptosis in PANC-1 cells and SW1990 cells. (A) Cell apoptosis rate of PANC-1 cells treated with PBS (control), LP, GPC1-LP, GEM, LP (GEM) and GPC1-LP (GEM) by flow cytometry analysis. (B) The expression of apoptosis-related proteins (Bcl-2, Bax, cleaved-caspase-3, and cleaved-caspase-9) in PANC-1 cells and SW1990 cells treated with PBS (control), GEM, LP (GEM) and GPC1-LP (GEM) by western blotting. *p<0.05.
GEM, LP (GEM) and GPC1-LP (GEM), was in comparison to that of the orthotopic PDAC mice (Figure 5A). Notably, the tumor weight was similar in control cells, cells with LP, and cells with GPC1-LP, while the tumor weight was lowest in cells with GPC1-LP (GEM), followed by cells with LP (GEM) and free GEM (Figure 5B), which suggested a better anti-tumor effect of GPC1-LP (GEM) on orthotopic PDAC mice.

The correlation between GPC1 mRNA and hypoxia in pancreatic cancer was analyzed by TCGA database, the result showed that GPC1 level was positive correlation with hypoxia (Figure 5C). Moreover, we detected the mRNA expression of the hypoxic-related genes in tumor tissues through qRT-PCR, and found that GPC1-LP (GEM) could significantly inhibit the expression of the hypoxic-related genes (Figure 5D).

Figure 5. GPC1-LP (GEM) inhibited tumor growth of the orthotopic pancreatic cancer mice. (A) The weight changes of the orthotopic pancreatic cancer mice treated with saline (control), LP, GPC1-LP, GEM, LP (GEM) and GPC1-LP (GEM). (B) The tumor weight of the orthotopic pancreatic cancer mice treated with saline (control), LP, GPC1-LP, GEM, LP (GEM) and GPC1-LP (GEM). (C) The correlation between GPC1 mRNA and hypoxia in pancreatic cancer was detected by TCGA database. (D) qRT-PCR was used to detect mRNA expression levels of tumor tissues and genes associated with hypoxia. *p<0.05.
DISCUSSION

In the present study, we successfully prepared LP (GEM) and GPC1-LP (GEM) with a successful GEM release within 24 h. Compared with free GEM, both LP (GEM) and GPC1-LP (GEM) significantly diminished cell viability and clone number as well as induced cell apoptosis in PANC-1 cells. Furthermore, both LP (GEM) and GPC1-LP (GEM) had no effects on body weight of orthotopic PDAC mice, while obviously reduced the tumor size. GPC1-LP (GEM) especially showed improved anti-cancer effect compared with LP (GEM) in vitro and in vivo.

As one of the common chemotherapeutic drugs, GEM has been widely applied for the clinical treatments of various cancers, including non-small cell lung cancer [16], metastatic triple-negative breast cancer [17], advanced ovarian cancer [18], bladder cancer [19], and PDAC [20]. In this study, free GEM significantly inhibited cell viability and clone number as well as increased the rate of apoptotic cells in PANC-1 cells. In addition, free GEM treatment also distinctly inhibited Bcl-2 level as well as promoted the expression of Bax, cleaved-caspase-3 and cleaved-caspase-9. It is well known that cell apoptosis is mainly regulated contribute to by mitochondria-mediated apoptosis pathway in cancers [21, 22]. As a downstream molecule of apoptosis pathway, caspase-3 can inhibit the ratio of Bcl-2/Bax and then contribute to cell apoptosis [22, 23]. In addition, caspase-3 also can be activated followed by the recruitment and activation of caspase-9 [21]. These results were supported by previous clinic trials [24, 25].

Unfortunately, the majority of researches have reported the modest prognostic effect of GEM in PDAC patients, and the poor outcomes are mainly caused by the minimal penetration of GEM in the targeted tissues and cells due to its unfavorable pharmacokinetic and pharmacodynamic profile and poor residence time [20, 26]. In addition, GEM combined with other targeted therapeutics also shows the limited improvement in clinical outcomes for PDAC patients [20, 26]. Thus, due to high biocompatibility, ability to carry large drug payloads, LPs are widely applied in cancer treatment as a promising drug delivery system [11]. Chang et al. have reported a Phase I trial that irinotecan-loaded LP can be applied for the treatment of patients with advanced refractory solid tumors [27]. Another phase I trial has also shown the therapeutic effect of camptothecin-loaded LP in patients with ovarian cancer [28]. In particular, Calvagno et al. have demonstrated that LP (GEM) showed higher antitumoral efficacy than free GEM in a colon carcinoma cell line [29]. Similar results are reported in vitro studies that reveal the significant reduction of cell viability in PDAC cells following LP (GEM) treatment compared with free GEM [30]. Moreover, in vivo experiments, LP (GEM) treatment significantly reduces the tumor size and volume in anaplastic thyroid carcinoma mice [31]. Bornmann et al. have also demonstrated that LP (GEM) has the increased antitumoral and antimetastatic activities compared with free GEM in orthotopic PDAC mice [32]. Consistent with these results, our study also revealed that compared with free GEM, LP (GEM) significantly diminished cell proliferation and induced cell apoptosis in PANC-1 cells, as well as obviously reduced the tumor size of orthotopic PDAC mice. Interestingly, our study found that higher dose of GEM released from both LP (GEM) and GPC1-LP (GEM) within 24 h at pH 5.0 (tumor endocytic compartment) than that at pH 7.4 (blood plasma), which indicated that more GEM was released under the tumor environment. These results confirmed the preferable anti-tumor effects of LP (GEM) than GEM in vitro and in vivo.

Furthermore, to enhance the accuracy of LP (GEM) targeting to PDAC, it is necessary to modify LP (GEM) by loading a targeted molecule specific to PDAC. Specific biomarkers should be highly expressed in the lesion tissues of PDAC, but absent or under-expressed in normal tissues. Unfortunately, many biomarkers, including CEA, CA242, and CA19-9, exhibited low specificity and sensitivity for the early diagnosis of PDAC [33–35]. Thus, PDAC biomarkers with high specificity and sensitivity are imperative to discover. Recently, more attention has focused on GPC1, which is considered as a potential and specific biomarker for PDAC [15]. Consistently, our study confirmed that GPC1 was specifically and highly expressed in PANC-1 cells. GPC1, as a transmembrane heparan sulfate proteoglycan, is involved in the cell proliferation, invasion, metastasis and apoptosis during the progression of PDAC [36, 37]. Thus, this study prepared GPC1-LP (GEM), and GPC1-LP (GEM) treatment further inhibited cell proliferation and promoted cell apoptosis in PANC-1 cells, as well as suppressed the tumor size of orthotopic PDAC mice, compared with LP (GEM). These results indicated that GPC1-LP (GEM) possessed enhanced anti-tumor effects than LP (GEM) and free GEM.

It’s definitely worth that our study demonstrates the enhanced anti-tumor activity of GPC1-LP (GEM) in PDAC both in vitro and in vivo. However, several limitations still exist in this study. First, in vitro and in vivo PDAC models may not be a perfect fit. In vivo study, only one PDAC cells line (PANC-1 cells) was used as PDAC model cell in the in vitro study. For another, the target-binding affinity of GPC1-LP (GEM) was not investigated in this study. Thus, anti-tumor effect of GPC1-LP (GEM) should be verified in more PDAC cell lines, and the targeted therapeutic efficiency
of GPC1-LP (GEM) should be explored by in vitro cell fluorescence and in vivo magnetic resonance imaging in future researches.

In conclusion, this study successfully developed GPC1-LP (GEM), and GPC1-LP (GEM) had a superior anti-tumor activity than LP (GEM) in PDAC in vitro and in vivo. Overall, GPC1-LP (GEM) might be a promising therapeutic nanomedicine in PDAC.

CONCLUSIONS

In conclusion, this study successfully developed GPC1-LP (GEM), and GPC1-LP (GEM) had a superior anti-tumor activity than LP (GEM) in PDAC in vitro and in vivo. Overall, GPC1-LP (GEM) might be a promising therapeutic nanomedicine in PDAC.

MATERIALS AND METHODS

Preparation of GPC1-LP (GEM)

LP (GEM) was firstly prepared. In brief, 120 mg of hydrogenated soybean phospholipid (HSPC) and 34 mg of cholesterol were dissolved in 6 mL of chloroform, and 2 mL of GEM hydrochloride solution (3 mg/mL) was added into the above lipid solution. Subsequently, a uniform emulsion was formed by the ultrasonic shaking for 10 min, and then chloroform was removed by reduced pressure distillation at 45°C for 1 h. After the temperature was raised to 60°C, 2 mL of isothermal PBS solution was added to hydrate for 1 h, followed by ultrasonication for 3 min, and repeated freeze-thaw for 4 cycles. Lastly, LP (GEM) was obtained by incubating with 12 mg of carboxylated distearoyl phospho-ethanolamine-polyethylene glycol (DSPE-PEG2000) for 15 min in a 60°C water bath. Similarly, unloaded LPs were prepared as described above but without the addition of GEM.

Afterwards, 900 μL of LP (GEM) solution was incubated with 100 μL of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (10 mg/mL) and 50 μL of N-Hydroxysuccinimide (10 mg/mL) by shaking at 400 rpm in a constant temperature shaker at 25°C for 3 h, then, 10 μL of anti-GPC1 antibody (10 nmol, Abcam, Cambridge, MA, USA) was added dropwise into the above mixture. After shaking for 2 h, the mixture was blocked with bovine serum albumin (BSA), and GPC1-LP (GEM) was obtained by shaking for 10 h.

Material characteristics

The size distribution of GPC1-LP (GEM) were observed by dynamic light scattering (DLS). DLS was performed by Zetasizer Nano Z (Worcestershire, UK).

In vitro drug release analysis

The drug release profiles of LP (GEM) and GPC1-LP (GEM) at different pH values were analyzed. In brief, 200 μL LP (GEM) and GPC1-LP (GEM) were separately loaded into a dialysis bag (molecular retention of 8,000-12,000 Da), and the dialysis bag was immersed in 35 mL of PBS buffer (pH 7.4 and 5.0, respectively). PBS buffer with pH 7.4 simulated blood plasma environment and pH 5.0 simulated tumor endocytic compartment. The entire dialysis system was shaken at 200 rpm in a constant temperature shaker at 37°C in the dark. Subsequently, 1 mL of dialysate was taken at 0.5 h, 1 h, 2 h, 4 h, 6 h, 9 h, 12 h, and 24 h, respectively. Finally, the concentration of GEM in the dialysate was determined by UV spectrophotometer, and the in vitro release profile was calculated.

Cell culture

Human pancreatic cancer cell line PANC-1 and human embryonic kidney cell line 293T were obtained from Shanghai Obio Technology Co., Ltd (China), and maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco) with standard incubation conditions (5% CO2 and 37°C).

Cell immunofluorescence

Expression of GPC1 was detected in PANC-1 and 293T cells. PANC-1 and 293T cells were fixed in 4% paraformaldehyde overnight, respectively. Then cells were incubated with GPC1 antibody, followed by the incubation of FITC-labelled secondary antibody. Cell nucleus was stained with DAPI for 5 min. Meanwhile, cells without FITC-labelled secondary antibody were served as control group. Last, inversion fluorescence microscope was used to observe the expression of GPC1.

MTT assay

PANC-1 and 293T cells were grown in 96-well plates for 24 h, and then incubated with GEM, LP (GEM) and GPC1-LP (GEM) BTZ (with GEM concentrations of 2, 5, 10, 20, 50 and 100 μg/mL), respectively, for 24 h and 48 h. Next, MTT (10 μL, Sigma, St Louis, MI, USA) was added to incubate with cells for 4 h, and dimethyl sulfoxide (150 μL, Sigma) was then used to dissolve formazan precipitates. The zero hole (medium, MTT, DMSO) and blank hole were set up. The absorbance at 450 nm were read by microplate reader (Molecular Devices, USA).
Colony formation assay

PANC-1 and 293T cells were grown in 96-well plates for 24 h, and then incubated with PBS (control), GEM, LP (GEM) and GPC1-LP (GEM) BTZ (with GEM concentrations of 10 μg/mL), respectively, for 24 h. Next, 0.5% (w/v) crystal violet in ethanol was added into cells for 5 min. The mean number of colonies was calculated under 10 different fields of vision.

Cell apoptosis assay

 Annexin V-FITC Apoptosis Detection kit was used to evaluate the cell apoptosis. PANC-1 cells were treated with PBS (control), LP, GPC1-LP, GEM, LP (GEM) and GPC1-LP (GEM) BTZ (with GEM concentrations of 10 μg/mL), respectively, for 24 h. Next, cells were digested with Trypsin and washed with PBS, followed by resuspending in 1 x Binding Buffer, and stained with PI and FITC-Annexin V for 15 min at 25°C in the dark. Cells were finally detected using flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Quantitative reverse transcription (qRT)-PCR

Total RNA from PANC-1 and 293T cells was obtained by Trizol (Invitrogen), respectively, and then reverse transcription of RNA was performed using PrimeScript™ RT reagent Kit (Takara, Dalian, China). The qRT-PCR was carried out by the SYBR Premix Ex Taq TM II (Takara) on Rotor- Gene RG-3000A (Corbett Life Science, Sydney, Australia). The PCR primers for GPC1 sense primer was 5′-TACAGAGGGCTCTCA AAGC-3′ and antisense primer was 5′-GGCATCATG CATCATCCTCAG-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer was 5′-GTG GATCAGCAAGCGAGT-3′ and antisense primer was 5′-AAGGCGATGCAATTCATC-3′. The PCR parameters were set as follows: 95°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 15 s. GAPDH were served as the internal control, and data were analyzed with 2−ΔΔCt method.

Western blotting assay

PANC-1 cells were treated with PBS (control), GEM, LP (GEM) and GPC1-LP (GEM) (with GEM concentrations of 10 μg/mL), respectively, for 24 h. Total proteins were obtained using lysis buffer, and then quantitated by bicinchoninic acid kit (Beyotime, Shanghai, China). Following sample separating and transferring into PVDF membranes, membranes were immersed in 5% nonfat milk for 1 h. Next, primary antibodies of GPC1, apoptosis-related proteins (including Bcl-2, Bax, cleaved-caspase-3 and cleaved-caspase-9) (1: 800, Abcam), as well as β-actin (1: 1000, Beyotime), respectively, were used for immunoblotting of the membranes overnight at 4°C. Then, membranes were reacted with secondary antibody (1: 1000, Beyotime) for 2 h keeping in dark at room temperature. The signals were revealed using enhanced chemiluminescence Plus reagent (Beyotime) to image blots. The band quantification was carried out using Image J software.

Animal model

Approval from the local animal Ethics Committee of the animal laboratory center of Zhengzhou University was obtained prior to experiments. Healthy male BALB/c nude mice (4 weeks old and weighing 18-22g, purchased from Charles River, Beijing, China) were used for the following experiments after one week of acclimation. Orthotopic PDAC model was established as previously described [38]. Briefly, PANC-1 cells were suspended in RPMI-1640 medium, and then 5 × 10^6 PANC-1 cells were subcutaneously injected into mice. After 4 weeks, the tumor could reach to about 8 mm in diameter. Next, tumors were resected into fragments (about 1 mm³), and then transplanted into the pancreas of nude mice. Orthotopic PDAC model was successfully obtained when the diameter of tumor reached to 5-10 mm.

In vivo therapeutic performance

A total of 48 BALB/c nude mice with orthotopic PDAC were randomly and equally divided into 6 groups, and treated, respectively, with saline (control), LP, GPC1-LP, GEM, LP (GEM) and GPC1-LP (GEM) by intravenous injection twice a week for 2 weeks. On day 15, the bodyweight of mice was monitored every 3 days to plot body weight curve and the mice were euthanized. In addition, the tumor was resected and tumor weight was measured.

Statistical analysis

Statistical analysis was carried out using statistical analysis software (SPSS 19.0, SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± S.D and analyzed by one-way analysis of variance. A P-value of < 0.05 was considered to indicate a statistically significant result.

AUTHOR CONTRIBUTIONS

Yu Mu and Dezhi Wang performed the majority of experiments, collected and analyzed the data; Liangyu Bie and Suxia Luo performed the molecular investigations; Xiaqian Mu designed and coordinated the research; Yanqiu Zhao wrote the paper.
CONFLICTS OF INTEREST

The author reports no conflicts of interest in this work.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. Lancet. 2016; 388:73–85. https://doi.org/10.1016/S0140-6736(16)00141-0 PMID:26830752

2. Ilic M, Ilic I. Epidemiology of pancreatic cancer. World J Gastroenterol. 2016; 22:9694–705. https://doi.org/10.3748/wjg.v22.i44.9694 PMID:27956793

3. Neesse A, Bauer CA, Öhlund D, Lauth M, Buchholz M, Michi P, Tuveson DA, Gress TM. Stromal biology and therapy in pancreatic cancer: ready for clinical translation? Gut. 2019; 68:159–71. https://doi.org/10.1136/gutjnl-2018-316451 PMID:30177543

4. Garrido-Laguna I, Hidalgo M. Pancreatic cancer: from state-of-the-art treatments to promising novel therapies. Nat Rev Clin Oncol. 2015; 12:319–34. https://doi.org/10.1038/nrclinonc.2015.53 PMID:25824606

5. Ansari D, Gustafsson A, Andersson R. Update on the management of pancreatic cancer: surgery is not enough. World J Gastroenterol. 2015; 21:3157–65. https://doi.org/10.3748/wjg.v21.i11.3157 PMID:25805920

6. Amrutkar M, Gladhaug IP. Pancreatic cancer chemoresistance to gemcitabine. Cancers (Basel). 2017; 9:157. https://doi.org/10.3390/cancers9110157 PMID:29144412

7. Neoptolemos JP, Palmer DH, Ghanen P, Psarelli EE, Valle JW, Halloran CM, Faluji O, O’Reilly DA, Cunningham D, Wadley J, Darby S, Meyer T, Gillmore R, et al, and European Study Group for Pancreatic Cancer. Comparison of adjuvant gemcitabine and capetitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. Lancet. 2017; 389:1011–24. https://doi.org/10.1016/S0140-6736(16)32409-6 PMID:28129987

8. Goldstein D, El-Maraghi RH, Hammel P, Heinemann V, Kunzmann V, Sastre J, Scheithauer W, Siena S, Tabernerio J, Teixeira L, Tortora G, Van Laethem JL, Young R, et al. Nab-paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial. J Natl Cancer Inst. 2015; 107:dju413. https://doi.org/10.1093/jnci/dju413 PMID:25638248

9. Catenacci DV, Juntilta MR, Karrison T, Bahary N, Horiba MN, Nattam SR, Marsh R, Wallace J, Kozloff M, Rajdev L, Cohen D, Wade J, Sleckman B, et al. Randomized phase Ib/II study of gemcitabine plus placebo or vismodegib, a hedgehog pathway inhibitor, in patients with metastatic pancreatic cancer. J Clin Oncol. 2015; 33:4284–92. https://doi.org/10.1200/JCO.2015.62.8719 PMID:26527777

10. de Sousa Cavalcante L, Monteiro G. Gemcitabine: metabolism and molecular mechanisms of action, sensitivity and chemoresistance in pancreatic cancer. Eur J Pharmacol. 2014; 741:8–16. https://doi.org/10.1016/j.ejphar.2014.07.041 PMID:25084222

11. Zununi Vahed S, Salehi R, Davaran S, Sharifi S. Liposome-based drug co-delivery systems in cancer cells. Mater Sci Eng C Mater Biol Appl. 2017; 71:1327–41. https://doi.org/10.1016/j.msec.2016.11.073 PMID:27987688

12. Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, Hua S. Advances and challenges of liposome assisted drug delivery. Front Pharmacol. 2015; 6:286. https://doi.org/10.3389/fphar.2015.00286 PMID:26648870

13. Matsumoto T, Kitahashi T, Komori T, Kitahara H, Ono K, Yamada N, Iwamura H, Takada K, Hagiwara S, Shimada Y. Abstrat 5148: Liposomal gemcitabine, FF-10832, improves gemcitabine (GEM) pharmacokinetics (PK) and increases anti-tumor efficacy. Cancer Res. 2017 (Supp); 77:5148. https://doi.org/10.1158/1538-7445.AM2017-5148

14. Kim DH, Im BN, Hwang HS, Na K. Gemcitabine-loaded DSPE-PEG-PheoA liposome as a photomediated immune modulator for cholangiocarcinoma treatment. Biomaterials. 2018; 183:139–50. https://doi.org/10.1016/j.biomaterials.2018.08.052 PMID:30170256

15. Diamandis EP, Plebani M. Glypican-1 as a highly sensitive and specific pancreatic cancer biomarker. Clin Chem Lab Med. 2016; 54:e1–2. https://doi.org/10.1515/cclm-2015-0773 PMID:26389634
16. Leijen S, Burgers SA, Baas P, Pluim D, Tibben M, van Werkhoven E, Alessio E, Sava G, Beijnen JH, Schellens JH. Phase I/II study with ruthenium compound NAMI-a and gemcitabine in patients with non-small cell lung cancer after first line therapy. Invest New Drugs. 2015; 33:201–14. https://doi.org/10.1007/s10637-014-0179-1 PMID:25344453

17. Hu XC, Zhang J, Xu BH, Cai L, Ragaz J, Wang ZH, Wang BY, Teng YE, Tong ZS, Pan YY, Yin YM, Wu CP, Jiang ZF, et al. Cisplatin plus gemcitabine versus paclitaxel plus gemcitabine as first-line therapy for metastatic triple-negative breast cancer (CBCSG006): a randomised, open-label, multicentre, phase 3 trial. Lancet Oncol. 2015; 16:436–46. https://doi.org/10.1016/S1470-2153(15)72004-1 PMID:25795409

18. Gray HJ, Bell-McGuinn K, Fleming GF, Cristea M, Xiong H, Sullivan D, Luo Y, McKee MD, Munasinghe W, Martin LP. Phase I combination study of the PARP inhibitor veliparib plus carboplatin and gemcitabine in patients with advanced ovarian cancer and other solid Malignancies. Gynecol Oncol. 2018; 148:507–14. https://doi.org/10.1016/j.ygyno.2017.12.029 PMID:29352572

19. Narayan V, Mamtani R, Keefe S, Guzzo T, Malkowicz SB, Vaughn DJ. Cisplatin, gemcitabine, and lapatinib as neoadjuvant therapy for muscle-invasive bladder cancer. Cancer Res Treat. 2016; 48:1084–91. https://doi.org/10.4143/crt.2015.405 PMID:26639198

20. Conroy T, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, Choné L, Francois E, Artru P, Biagi JJ, Lecomte T, Assenat E, Faroux R, et al, and Canadian Cancer Trials Group and the Unicancer-GI-PRODIGE Group. FOLFIRINOX or gemcitabine as adjuvant therapy for pancreatic cancer. N Engl J Med. 2018; 379:2395–406. https://doi.org/10.1056/NEJMoa1809775 PMID:30575490

21. Lopez J, Tait SW. Mitochondrial apoptosis: killing cancer using the enemy within. Br J Cancer. 2015; 112:957–62. https://doi.org/10.1038/bjc.2015.85 PMID:25742467

22. Ichim G, Tait SW. A fate worse than death: apoptosis as an oncogenic process. Nat Rev Cancer. 2016; 16:539–48. https://doi.org/10.1038/nrc.2016.58 PMID:27364482

23. Hatok J, Racay P. Bcl-2 family proteins: master regulators of cell survival. Biomol Concepts. 2016; 7:259–70. https://doi.org/10.1515/bmc-2016-0015 PMID:27505095

24. Golcher H, Brunner TB, Witzigmann H, Marti L, Bechstein WO, Bruns C, Jungnickel H, Schreiber S, Grabenbauer GG, Meyer T, Merkel S, Fietkau R, Hohenberger W. Neoadjuvant chemoradiation therapy with gemcitabine/cisplatin and surgery versus immediate surgery in resectable pancreatic cancer: results of the first prospective randomized phase II trial. Strahlenther Onkol. 2015; 191:7–16. https://doi.org/10.1007/s00066-014-0737-7 PMID:25252602

25. Middleton G, Palmer DH, Greenhalf W, Ghaneh P, Jackson R, Cox T, Evans A, Shaw VE, Wadsley J, Valle JW, Propper D, Wasan H, Falk S, et al. Vandetanib plus gemcitabine versus placebo plus gemcitabine in locally advanced or metastatic pancreatic carcinoma (ViP): a prospective, randomised, double-blind, multicentre phase 2 trial. Lancet Oncol. 2017; 18:486–99. https://doi.org/10.1016/S1470-2153(17)30084-0 PMID:28259610

26. Gillen S, Schuster T, Meyer Zum Büschenfelde C, Friess H, Kleeff J. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLoS Med. 2010; 7:e1000267. https://doi.org/10.1371/journal.pmed.1000267 PMID:20422030

27. Chang TC, Shiah HS, Yang CH, Yeh KH, Cheng AL, Shen BN, Wang YW, Yeh CG, Chiang NJ, Chang JY, Chen LT. Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients. Cancer Chemother Pharmacol. 2015; 75:579–86. https://doi.org/10.1007/s00280-014-2671-x PMID:25577133

28. Zamboni WC, Ramalingam S, Friedland DM, Edwards RP, Stoller RG, Strychor S, Maruca L, Zamboni BA, Belani CP, Ramanathan RK. Phase I and pharmacokinetic study of pegylated liposomal CKD-602 in patients with advanced Malignancies. Clin Cancer Res. 2009; 15:1466–72. https://doi.org/10.1158/1078-0432.CCR-08-1405 PMID:19190127

29. Calvagno MG, Celia C, Paolino D, Cosco D, Iannone M, Castelli F, Doldo P, Frest M. Effects of lipid composition and preparation conditions on physical-chemical properties, technological parameters and in vitro biological activity of gemcitabine-loaded liposomes. Curr Drug Deliv. 2007; 4:89–101. https://doi.org/10.2174/15672010779314749 PMID:17269921
30. Cosco D, Bulotta A, Ventura M, Celia C, Calimeri T, Perri G, Paolino D, Costa N, Neri P, Tagliaferri P, Tassone P, Fresta M. In vivo activity of gemcitabine-loaded PEGylated small unilamellar liposomes against pancreatic cancer. Cancer Chemother Pharmacol. 2009; 64:1009–20. https://doi.org/10.1007/s00280-009-0957-1 PMID: 19263052

31. Paolino D, Cosco D, Racanicchi L, Trapasso E, Celia C, Iannone M, Puxeddu E, Costante G, Filetti S, Russo D, Fresta M. Gemcitabine-loaded PEGylated unilamellar liposomes vs GEMZAR: biodistribution, pharmacokinetic features and in vivo antitumor activity. J Control Release. 2010; 144:144–50. https://doi.org/10.1016/j.jconrel.2010.02.021 PMID: 20184929

32. Bornmann C, Graeser R, Esser N, Ziroli V, Jantscheff P, Keck T, Unger C, Hopt UT, Adam U, Schaechtele C, Massing U, von Dobschuetz E. A new liposomal formulation of gemcitabine is active in an orthotopic mouse model of pancreatic cancer accessible to bioluminescence imaging. Cancer Chemother Pharmacol. 2008; 61:395–405. https://doi.org/10.1007/s00280-007-0482-z PMID: 17554540

33. Houghton JL, Zeglis BM, Abdel-Atti D, Aggeler R, Sawada R, Agnew BJ, Scholz WW, Lewis JS. Site-specifically labeled CA19.9-targeted immunoconjugates for the PET, NIRF, and multimodal PET/NIRF imaging of pancreatic cancer. Proc Natl Acad Sci USA. 2015; 112:15850–55. https://doi.org/10.1073/pnas.1506542112 PMID: 26668398

34. Chen Y, Gao SG, Chen JM, Wang GP, Wang ZF, Zhou B, Jin CH, Yang YT, Feng XS. Serum CA242, CA199, CA125, CEA, and TSGF are biomarkers for the efficacy and prognosis of cryoablation in pancreatic cancer patients. Cell Biochem Biophys. 2015; 71:1287–91. https://doi.org/10.1007/s12013-014-0345-2 PMID: 25486903

35. Xu HX, Li S, Wu CT, Qi ZH, Wang WQ, Jin W, Gao HL, Zhang SR, Xu JZ, Liu C, Long J, Xu J, Ni QX, et al. Postoperative serum CA19-9, CEA and CA125 predicts the response to adjuvant chemoradiotherapy following radical resection in pancreatic adenocarcinoma. Pancreatology. 2018; 18:671–77. https://doi.org/10.1016/j.pan.2018.05.479 PMID: 30153903

36. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, LeBleu VS, Mittendorf EA, Weitz J, Rahbari N, Reissfelder C, Pilarsky C, Fraga MF, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nature. 2015; 523:177–82. https://doi.org/10.1038/nature14581 PMID: 26106858

37. Herreros-Villanueva M, Bujanda L. Glypican-1 in exosomes as biomarker for early detection of pancreatic cancer. Ann Transl Med. 2016; 4:64. https://doi.org/10.3978/j.issn.2305-5839.2015.10.39 PMID: 27004211

38. Qiu W, Zhang H, Chen X, Song L, Cui W, Ren S, Wang Y, Guo K, Li D, Chen R, Wang Z. A GPC1-targeted and gemcitabine-loaded biocompatible nanoplatform for pancreatic cancer multimodal imaging and therapy. Nanomedicine (Lond). 2019; 14:2339–53. https://doi.org/10.2217/nnm-2019-0063 PMID: 31414945