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Original article

Effect of gemcitabine and retinoic acid loaded PAMAM dendrimer-coated magnetic nanoparticles on pancreatic cancer and stellate cell lines

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ABSTRACT

Gemcitabine is an anticancer drug used in the treatment of different cancer types, including pancreatic ductal adenocarcinoma. The maximum tolerated dose in humans is restricted by its side effects on healty cells. Furthermore, the fibrotic stroma produced by the pancreatic stellate cells prevents effective delivery of chemotherapeutic agents providing a safe-haven for the cancer cells. This becomes more of a problem considering the short half-life of this drug. Magnetic nanoparticle-based targeted drug delivery systems are a promising alternative to overcome the limitations of classical chemotherapies. The aim of this study is to obtain an effective targeted delivery system for gemcitabine using magnetic nanoparticles (MNPs) and all-*trans* retinoic acid (ATRA). This dual approach targets the tumor cells and its infrastructure – stellate cells – simultaneously. Gemcitabine and ATRA were loaded onto the PAMAM dendrimer-coated magnetic nanoparticles (DcMNPs), which were synthesized and characterized previously. Drug loading and release characteristics, and stability of the nanoparticles were investigated. Gemcitabine and ATRA loaded MNPs are efficiently taken up by pancreatic cancer and stellate cells successfully targeting and eliminating both cells. Results of this study can provide new insights on pancreatic cancer therapy where tumor is seen as a system with its stroma insead of epithelial cells alone.

1. Introduction

Pancreatic cancer is a disease with extremely poor prognosis. The five-year survival rate has only improved marginally over the past decades, and remains less than 5% [1,2]. Pancreatic cancer has the lowest long-term survival rate among the common malignancies. The disease typically displays an aggressive, and infiltrative growth pattern, with metastases often already seeded at the time of diagnosis. Pancreatic cancer is characterized by an abundant fibrotic tumor stroma, referred to as the desmoplastic reaction, which surrounds and infiltrates clusters of cancer cells [3]. Pancreatic ductal adenocarcinoma (PDAC) is a desmoplastic tumour and has a propensity for early metastasis and high resistance to both chemo- and radiotherapy. In PDAC, the fibrotic stroma impacts on tumorigenesis, angiogenesis, therapy resistance and possibly the metastatic spread of tumor cells [4–6]. Recently, the abundant fibrotic stroma produced by the activated pancreatic stellate cells (PSCs) has attracted attention, as it forms a physical barrier for the effective delivery of therapeutic agents. To break this fibrotic wall, restoration of quiescence in activated pancreatic stellate cells and/or their elimination may offer a novel therapeutic strategy [7–9].

PSCs belong to the retinoid-storing cells, and in their quiescent (non-activated) states, retinol (vitamin A) is stored as lipid droplets in their cytoplasm. Activation of PSCs is invariably associated with loss of the retinol containing droplets from their cytoplasm [10]. Studies in other cell systems have established that retinol is an essential compound of normal cell biology [11]. In particular, metabolites of retinol, all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-RA), have been shown to mediate a number of cellular functions, including proliferation, differentiation, and protein synthesis [12].

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Chemotherapy is applied as neoadjuvant or adjuvant therapy for pancreatic cancer. Gemcitabine is the most commonly used first line therapy in pancreatic cancer. However it has severe side effects, such as anemia, leukopenia. Gemcitabine has a short half-life *in vivo* and will be rapidly and extensively decomposed to inactive products in the blood, liver, kidney, and other tissues [13]. In addition, multidrug resistance usually develops and prevents further therapy by gemcitabine. Recently the fibrotic stroma, found commonly in PDAC, is identified as a barrier for the effective delivery of chemotherapy [14–16]. Unfortunately PSC are highly resistant to gemcitabine [17]. As expected, the development of a suitable nanocarrier mediated drug delivery system that maximizes the therapeutic value of gemcitabine while minimizing its side effects has gained high attention [18].

Nanocarrier mediated drug delivery has emerged as a successful strategy to enhance delivery of therapeutics to tumors. This technology has enabled the development of nanosized particles that can be fabricated from a multitude of materials in a variety of compositions, including polymeric, dendrimeric, and magnetic nanoparticles (MNPs) [19]. Magnetic nanoparticles, which can be targeted to tumor site in a magnetic field, gained importance on cancer therapy in recent years. Magnetic nanoparticles have reactive surface that can be readily modified with biocompatible coatings and loaded with therapeutic agents [20,21]. Dendrimers are repetitively branched molecules, which are typically symmetric around the core and often adopt a spherical three-dimensional morphology [22,23]. Properties of these particles, such as water solubility, modifiable surface functionality, availability of internal cavities, and targeting efficiency under magnetic field makes them attractive for drug delivery applications. The aim of this study is three-fold:

- to show that gemcitabine and ATRA can be coupled together on MNPs;
- this compound can be uptaken in cancer- and stellate cells;
- it shows a dual antiproliferative effect on cancer as well as stellate cells.

In this double-hit strategy, we aim to use ATRA as bait to target the stromal component, while gemcitabine will serve as the standard chemotherapeutic agent.

2. Material and methods

2.1. Materials

Ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), 32% ammonia solution (NH_3), 3-aminopropyltrimethoxysilane (NH_2 $(CH_2)_3$–Si–$(OCH_3)_3$, APTS), methyl acrylate, methanol, ethanol, and ethylenediamine were purchased from Sigma–Aldrich.

Isolation and cultivation of PSC were performed as published before [24]. The usage of human material and isolation of primary cells were approved by the local ethics committee of the Technical University of Munich (Germany), and written informed consent was obtained from all patients. Human pancreatic cancer cell lines Panc-1, SU86.86, and T3M4 were purchased from ATCC (Rockville, MD, USA).

2.2. Preparation of magnetic nanoparticles

PAMAM dendrimer-coated DcMNPs were synthesized as explained by Khodadust et al., [25]. The MNPs (Fe_3O_4) were prepared with a minor modification of co-precipitation method. Surface modification of Fe_3O_4 was performed with 3-aminopropyltrimethoxysilane (APTS). Surface coating of G_0 generation of nanoparticles was carried out with PAMAM dendrimer through Michael reaction [26,27]. The characterization of PAMAM dendrimer-coated DcMNPs was performed by TEM, VSM, FT–IR, XRD analyses.

2.3. Gemcitabine and ATRA loading on dendrimer-coated magnetic nanoparticles (DcMNPs)

The first agent, ATRA, was conjugated in methanol at different concentrations (10–25 μM). Gemcitabine loading studies were carried out in PBS (pH 7.2) buffer, at different drug concentrations (10–25 μM). The mixture of buffer, gemcitabine and ATRA loading DcMNPs was rotated at 100 rpm with 5 s vibration intervals for 24 h in the light protected tubes. After the incubation period, ATRA and gemcitabine-loaded DcMNPs were separated by magnetic decantation. Gemcitabine and ATRA loading efficiency was quantified by measuring the absorbance values at 269 nm and 340 nm by a Shimadzu UV spectrophotometer, respectively. The loading of gemcitabine (10–25 μM) and ATRA to DcMNPs was confirmed by FT–IR analysis.

2.4. Release of gemcitabine and ATRA from DcMNPs

The release of gemcitabine (10 μM) and ATRA (10 μM) from DcMNPs was analyzed in acetate buffer (pH 4) up to 24 h. The amount of released gemcitabine and ATRA was determined by measuring the absorbance of the solution at 269 and 340 nm, respectively.

2.5. MTT cytotoxicity assay

The cytotoxicity assay was performed on pancreatic cancer cell lines (SU86.86, T3M4, Panc-1) and on primary human pancreatic stellate cells (PSC). PSC were grown in the standard medium 1:1 (vol/vol) mixture of low glucose (1000 mg/L) Dulbecco's modified Eagle medium with Ham's F12 medium supplemented with 10% FCS, L-glutamine (2 mmol/L), and 1% penicillin/streptomycin. Cancer cells were cultivated in RPMI-1640, supplemented with 10% FBS (fetal bovine serum; Sigma, USA) and 1% penicillin–streptomycin mixture. The growth inhibition was evaluated by 3-(4,5-di-methylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, M2128) cytotoxicity assay (as published before) [28]. Samples were treated with freshly prepared solutions of ATRA in dimethylsulphoxide (DMSO), gemcitabine in PBS, ATRA and gemcitabine-loaded DcMNPs. After an incubation period, 10 μL of a 5 mg/mL MTT solution in PBS (pH 7.4) was added to each well and the plate was incubated for 4 h at 37 °C, allowing viable cells to reduce the MTT into purple coloured formazan crystals. The formazan crystal was dissolved by adding 100 μL of acidic isopropanol buffer. The absorbance was measured at 570 nm using an ELISA plate reader (Promega, Glomax Multı Detection System). All experiments were repeated three times.

2.6. Cellular internalization of dendrimer-coated magnetic nanoparticles

The internalization of dendrimer-coated iron oxide nanoparticles were confirmed by light microscopy (Zeiss, city, country). The cancer (SU86.86, T3M4, Panc-1) and PSC-lines were incubated with nanoparticles in 6-well plates. After 24 h incubation, the medium was removed from the plates and the plates were washed with PBS for several times so that all free DcMNPs were removed from the environment. Representative photographs were taken using an inverted optical microscope to determine cellular internalizations of DcMNPs.

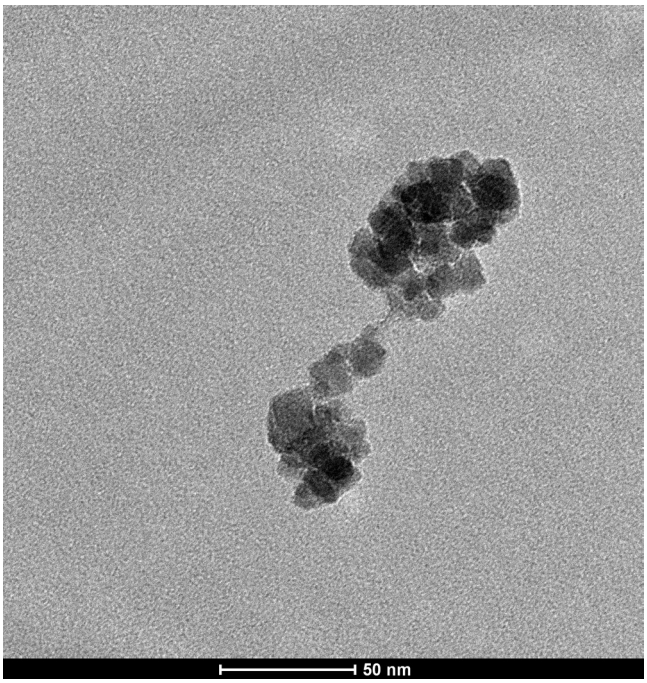

Fig. 1. The sizes of the obtained G4DcMNPs were 13 ± 5 nm.

3. Results

3.1. Characterization of dendrimer-coated magnetic iron oxide nanoparticles

Characterizations of dendrimer-coated MNPs were performed by XRD, XPS/ESCA, FT–IR, DLS, TGA analyses [25] and TEM (Fig. 1). XRD results demonstrated that the nanoparticles with preferred crystalline structure were obtained at 90 °C. The applied magnetic field was changed and magnetization properties of synthesized Fe_3O_4, aminosilane-modified MNPs, and DcMNPs were measured at 25 and 37 °C. Magnetic materials showing a superparamagnetic behavior have zero value of remanence and coercivity.

3.2. Fourier transform–infrared spectroscopy (FT–IR)

FT–IR results related to dendrimer-modified MNPs are given in Fig. 2. The characteristic new bonds of gemcitabine and ATRA were observed after loading dendrimer-coated MNPs. Fig. 2 shows the FT–IR spectra of native gemcitabine and ATRA with characteristic peaks at about 3396 cm^{-1}, 3260 cm^{-1}, 3115 cm^{-1}, 3077 cm^{-1}, 1671 cm^{-1}, 1535 cm^{-1}, 1286 cm^{-1}, 1201 cm^{-1}, 1065 cm^{-1} and 2932 cm^{-1}, 2866 cm^{-1}, 1689 cm^{-1}, 1251 cm^{-1}, 1182 cm^{-1}, respectively. After conjugation, the ATRA and gemcitabine-loaded MNPs, as shown in Fig. 3, displays the characteristic peaks at 3428 cm^{-1}, 2917 cm^{-1}, 2847 cm^{-1}, 1680 cm^{-1} and 1055 cm^{-1}.

3.3. Gemcitabine and ATRA loading efficiencies

Loading efficiencies were tested in PBS (pH 7.2) buffer with different drug concentrations. The loading efficiencies of 10 μM gemcitabine and ATRA were 83% and 70% in PBS buffer, respectively. To find the maximum drug loading capacity on DcMNPs, gemcitabine and ATRA concentration gradually increased up to 25 μM where the loading efficiency started to decrease. The highest and most efficient drug loading concentrations were obtained as 10 μM (Fig. 4).

3.4. Release efficiencies of gemcitabine and ATRA

Gemcitabine and ATRA release studies were also performed in acetate buffer having a pH 4.2 that mimics endosomal conditions. The release profile of DcMNPs at pH 4.2 is given in Fig. 5. The release studies were continued up to 22 h. Most of the drugs were released within the first 10 h. Release of gemcitabine at the first 10 h was approximately 72%, and release of ATRA was nearly 81%.

3.5. Cellular internalization of dendrimer-coated magnetic nanoparticles

Inverted light microscopy demonstrated that most of the DcMNPs were internalized at 37 °C in 4 h, while none of the bare MNPs is taken up by the cells. DcMNPs containing SU86.86, T3M4, Panc-1, PSC cells are shown in Figs. 6–9.

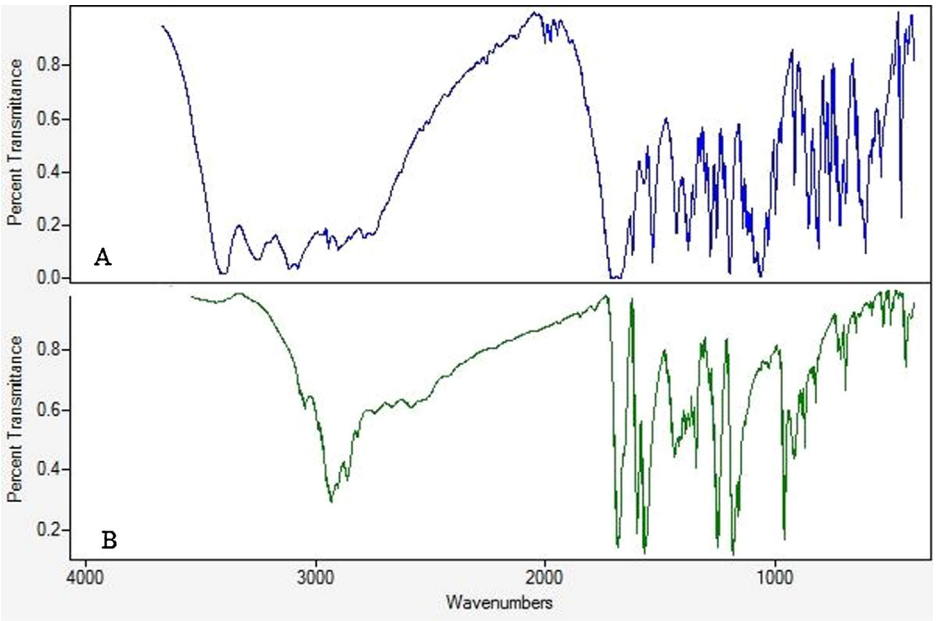

Fig. 2. FT–IR results related to (A) gemcitabine (B) ATRA.

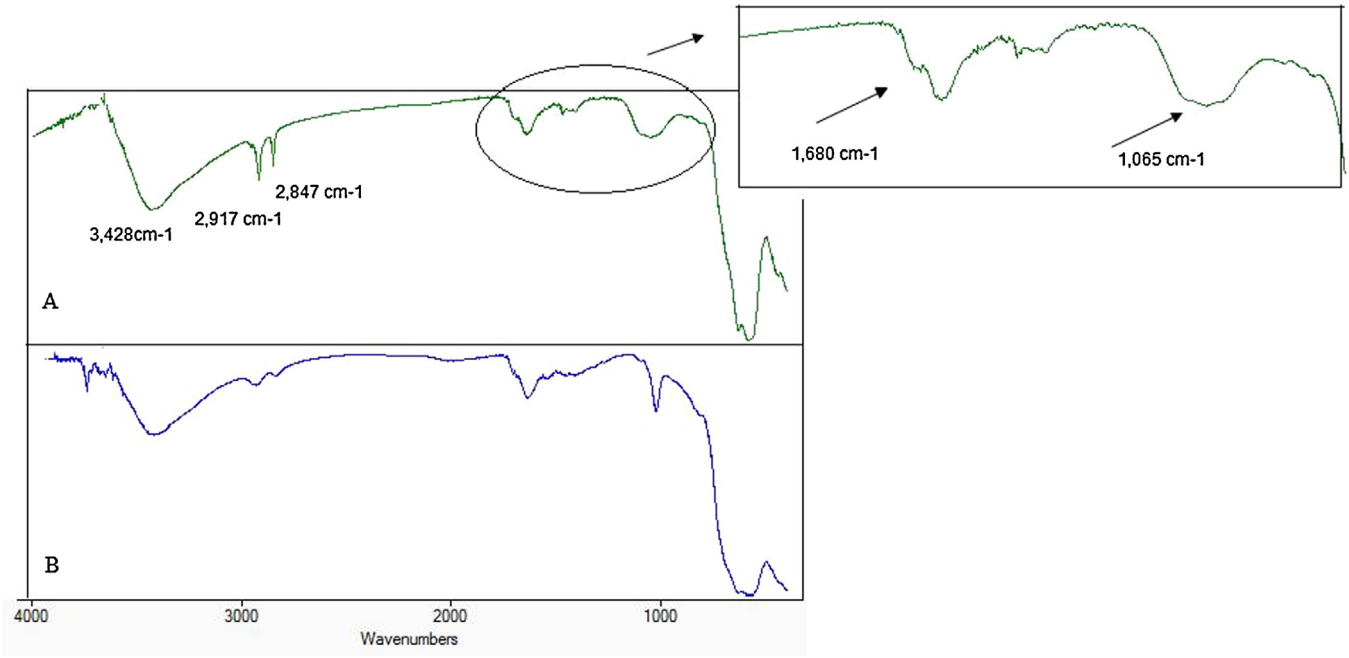

Fig. 3. FT–IR results related to (A) gemcitabine and ATRA loaded magnetic nanoparticles. B. Dendrimer-modified magnetic nanoparticles (DcMNPs).

3.6. Cytotoxicity analyses of gemcitabine and ATRA loaded dendrimer-coated magnetic nanoparticles

Cytotoxicity of DcMNPs, ATRA, gemcitabine and gemcitabine–ATRA loaded DcMNPs were investigated by MTT cell proliferation assay. Survival rates indicated that there is no significant cytotoxicity of the dendrimer-coated magnetic nanoparticles on SU86.86, T3M4, Panc-1 and PSC cells up to 250 μg/mL (Fig. 10).

It was found that the released gemcitabine and ATRA from the DcMNPs (100 μg/mL) has the capacity to kill SU86.86, T3M4, Panc-1, PSC cells efficiently (Fig. 11).

4. Discussion

In this study, we report that gemcitabine and retinoic acid loaded magnetic nanoparticles increase the effects on pancreatic cancer cell lines as well as PSC. As of today, there are no reports on the cytotoxic effects of retinoids combined with gemcitabine-loaded magnetic nanoparticles.

It has previously been reported that retinoids may play an important role to increase the cytotoxicity of various chemotherapeutic agents in various cancer types (lymphoma, leukemia, melanoma, lung cancer, cervical cancer, kidney cancer, neuroblastoma and glioblastoma). In the literature, studies indicated that lower vitamin A intake generates higher risk for cancer development [29,30]. The use of retinoids in combination with chemotherapeutic agents has been investigated, and reports suggested that retinoids can act to synergistically increase the cytotoxicity of drugs [31]. *In vivo* experiments showed that the combination was well tolerated by nude mice. On the other hand, retinoic acid has been reported to revert the activated myofibroblastic phenotype of PSC back to quiescence [32,33].

Gemcitabine is an anticancer drug, which requires cellular uptake and phosphorylation to active metabolites, which inhibit DNA chain elongation and lead to DNA fragmentation and cell death [34]. However, this anticancer drug has side effects on healthy cells and drug resistance leads to the failure of tumor treatment. Gemcitabine is metabolized by deoxycytidine kinase into its active di- and triphosphate forms, respectively [35,36]. Gemcitabine diphosphate is responsible for inhibiting

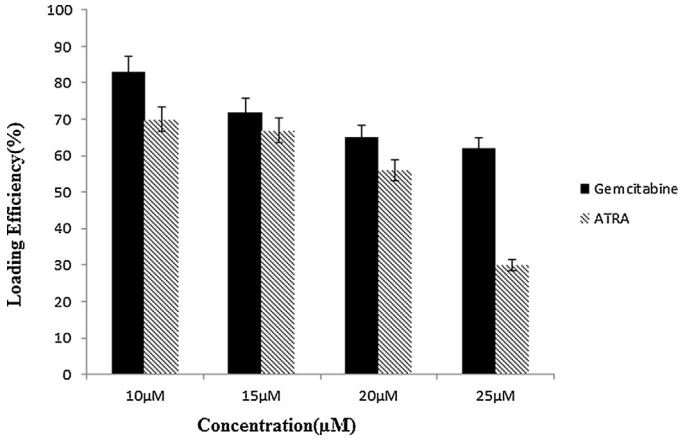

Fig. 4. Loading efficiencies of gemcitabine and ATRA to DcMNPs.

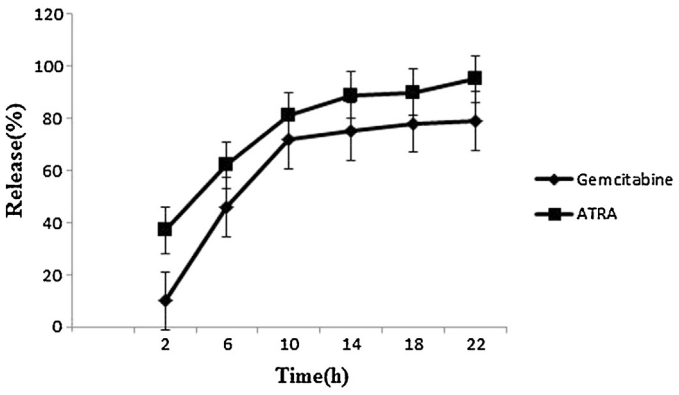

Fig. 5. Release efficiencies of gemcitabine and ATRA from DcMNPs.

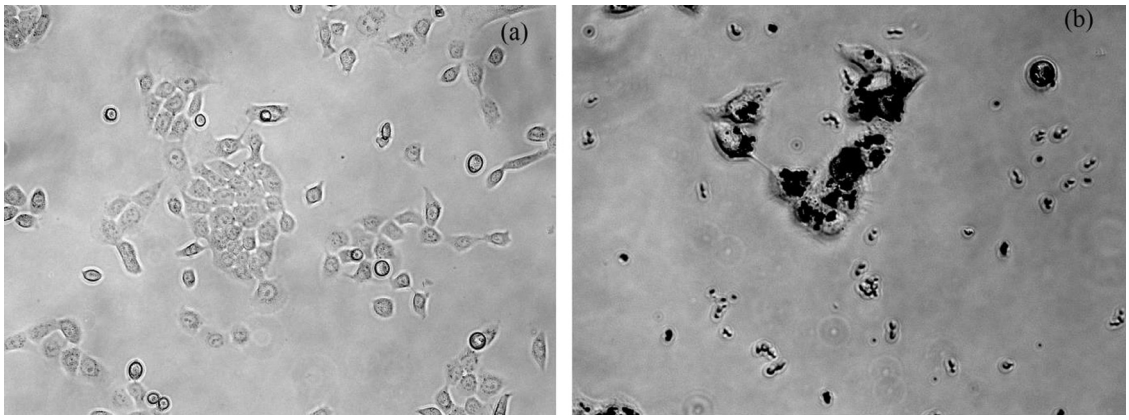

Fig. 6. Cellular internalization of dendrimer-coated magnetic nanoparticles in T3M4 cells by inverted light scattering microscopy (a) non-treated cells (control); b: cells treated with DcMNPs at 4 h.

ribonucleotide reductase and gemcitabine triphosphate incorporates into DNA, which leads to masked chain termination, decreased DNA synthesis [35,37,38]. Thus, in pancreatic cancer cells, we indicated gemcitabine and ATRA efficient action upon internalization after binding with MNPs.

Michael et al., [39] applied retinoic acid (RA) in combination with gemcitabine in patients with unresectable pancreatic carcinoma. The combination of gemcitabine and RA was well tolerated, but they did not see improvement in the response rate. The synergistic effects, *in vitro*, which were seen in pancreatic cancer cells, could not be fully confirmed in the xenograft model.

For the success of the drug-loaded delivery systems, the right balance between plasma stability and efficient drug release at the target site is critical. In this study, magnetic nanoparticles loaded with 10 μM gemcitabine and ATRA in PBS buffer, which releases most of the drug in lower pH in physiological condition, seems to

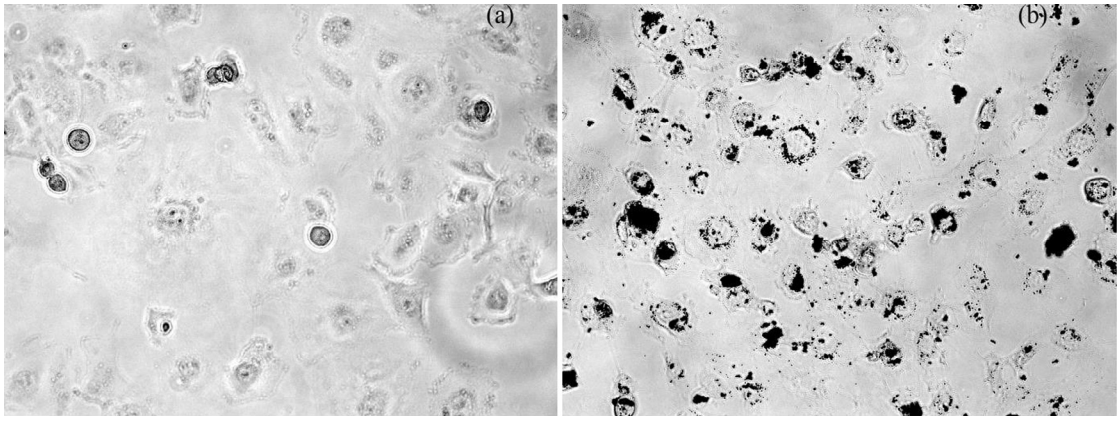

Fig. 7. Cellular internalization of dendrimer-coated magnetic nanoparticles In SU86.86 cells by inverted light scattering microscopy (a) non-treated cells (control): b: cells treated with DcMNPs at 4 h.

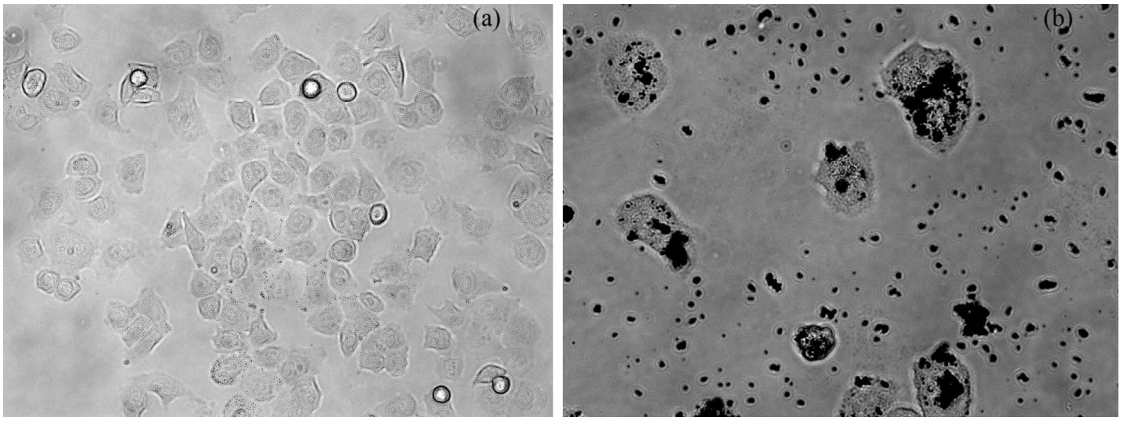

Fig. 8. Cellular internalization of dendrimer-coated magnetic nanoparticles In Panc-1 cells by inverted light scattering microscopy (a) non-treated cells (control); b: cells treated with DcMNPs at 4 h.

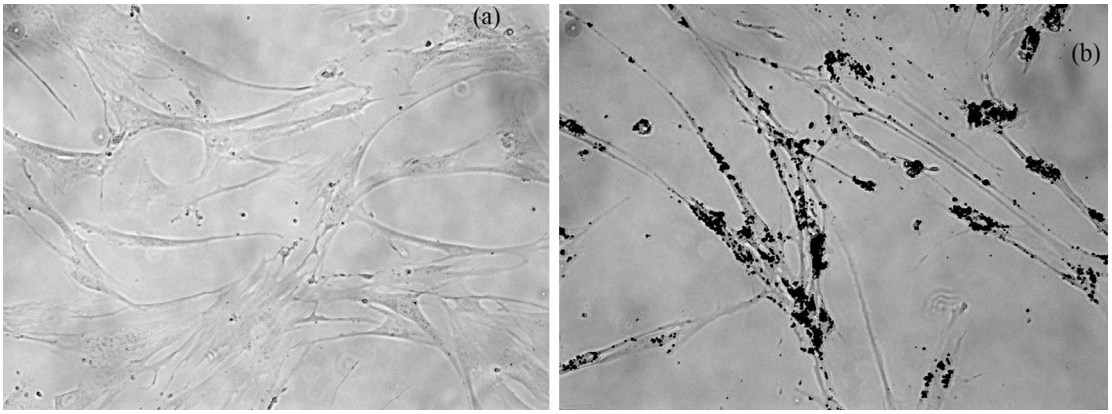

Fig. 9. In PSC cellular internalization of dendrimer-coated magnetic nanoparticles by inverted light scattering microscopy (a) non-treated cells (control); b: cells treated with DcMNPs at 4 h.

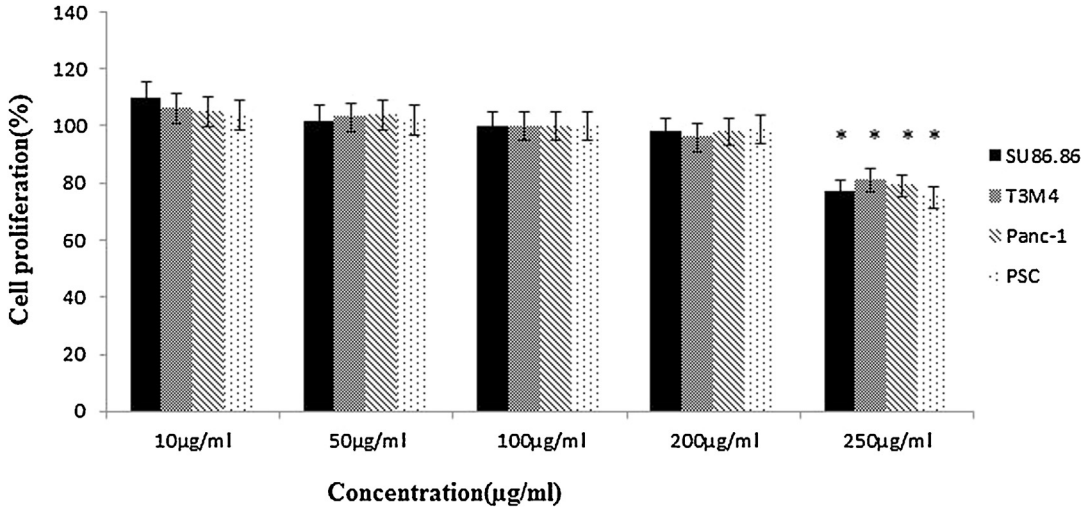

Fig. 10. Cytotoxicity analyses of dendrimer-coated magnetic nanoparticles in different concentrations on pancreatic cancer and stellate cells.

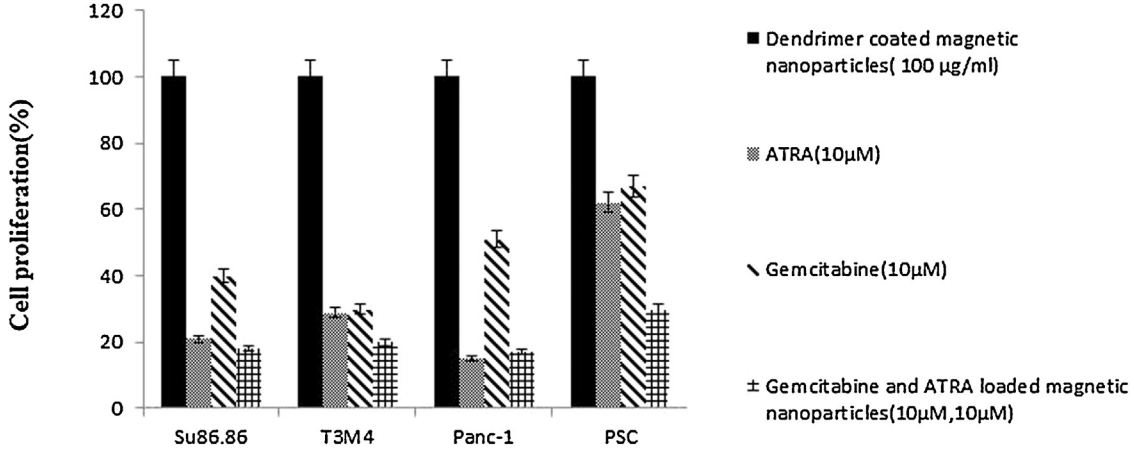

Fig. 11. Cytotoxicity analyses of DcMNPs, ATRA, gemcitabine, and gemcitabine–ATRA loaded DcMNPs on pancreatic cancer and stellate cells.

be the most suitable magnetic nanoparticles for efficient gemcitabine and ATRA delivery. The significant cell death confirms that the released drug is released and active.

The microscopy images demonstrated that gemcitabine and ATRA loaded MNPs were successfully taken up by pancreatic cancer and PSC cells. In addition, these nanoparticles lead to decrease in proliferation of cells. Application of gemcitabine and ATRA as loaded on MNPs caused the reversal of drug resistance by increasing the accumulation of drug in the cells. The application of gemcitabine and ATRA loaded magnetic nanoparticles may help to overcome resistance mechanism of gemcitabine resistant pancreatic cells. These results also show an unknown toxic effect of

ATRA on pancreatic cancer cell lines that deserves further elucidation.

5. Conclusion

The results of this study may provide new insights in pancreatic cancer therapy. Since PSC are shown to support tumor growth and increase therapy resistance, targeting tumor stroma (PSC), hence, its infrastructure may increase drug delivery to cancer cells.

Acknowledgments

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