Project Title

evelopment and Characterization of Polymeric Nanoparticulate Delivery System for Gemcitabine

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Review of the literature

Different groups of researchers have tested the efficacy of various available chemotherapeutic agents in combination with gemcitabine. The combination therapy did not seem to provide any significant benefits over the use of gemcitabine as a single agent in most cases [1]. Administration of a combination therapy of gemcitabine, tegafur and uracil in metastatic pancreatic cancer patients only showed moderate clinical benefits in a phase II trial [2]. Reni and coworkers investigated the usefulness of cisplatin, epirubicin and 5FU in combination with gemcitabine, the combination therapy was called PEFG. A higher percentage of one year overall survival was observed in the patient group receiving PEFG (38.5 % compared to 21.3 % in patients on gemcitabine alone). While, a significant increase in incidence of grade 3-4 neutropenia and thrombocytopenia was observed with PEFG [3].

The use of capecitabine along with gemcitabine has produced conflicting results in two different studies. In a phase III randomized trial, the patients receiving the combination therapy showed evidence of an increased median survival time with similar grade 3 and 4 toxicity profile [4]. Whereas, in another phase III trial by Cunningham et al., the use of gemcitabine alone was found to have a higher median survival time [5]. Different amounts of capecitabine were administered in these studies. The higher survival time with the combination therapy in the study could be attributed to a high amount of capecitabine administered to the patients [5]. These results have been shown in many other studies.

Gemcitabine is the most potent chemotherapeutic agent among its class of cytidine analogues. It has a dual mechanism of action; it causes cell cycle arrest at S phase. When transported into cells by nucleoside transporters, it is phosphorylated to difluoro deoxycytidine diphosphate (dfdCDP) and difluoro deoxycytidine triphosphate (dfdCTP). DfdCDP acts by inhibiting ribonucleotide reductase thus reducing the availability of deoxyribonucletides essential for DNA synthesis. On the other hand, dfdCTP competes with cytidine triphosphate (CTP) to get incorporated in to DNA and leads to DNA strand termination. A combination therapy comprising of oxiplatin, irinotecan, leucovorin followed by 5 FU (FOLFIRINOX) has also been tested against gemcitabine. The combination therapy showed slight improvement in survival time. In a recent study, Fine and group have explored the use of capecitabine, gemcitabine and docetaxel in combination for treating metastatic pancreatic cancer. The combination therapy produced significant results by extending the survival time to 11.2 months along with a 2-year survival rate of 20% [6, 7].

To date gemcitabine still remains the principal drug for the treatment of both locally advanced and metastatic pancreatic cancer. The standard dosing schedule for gemcitabine is 1-1.2 mg/m^2 as a 30-minute intravenous infusion on day 1, 8, 15 and 28 of every dosing cycle.

Even after exhibiting extremely promising antitumor activities against various kinds of tumors, the use of gemcitabine is limited to breast cancer, ovarian cancer, non-small cell lung cancer and pancreatic cancer. The most important drawback associated with the current clinical treatment with gemcitabine is its very short half-life. Gemcitabine is rapidly metabolized in plasma into dfdU by the enzyme cytidine deaminase. Therefore, it requires administration of high doses leading to dose-limiting adverse effects. Also, a very hydrophilic molecule gemcitabine requires assistance of nucleoside transporters to be transported into the cell. A deficiency of nucleoside transporters is the most common mechanism of development of resistance to gemcitabine. All these drawbacks necessitate an improved delivery system that is target specific and also possesses the ability to release the drug in a sustained fashion thus eliminating the systemic toxicity [8].

The goal of any drug delivery system is to achieve a therapeutic amount of the drug at the appropriate site in the body and also it should deliver the drug at a rate required to meet the needs of the body over time. Hence the two most important objectives of any delivery system are spatial placement and temporal delivery. An appropriately designed controlled delivery system is an ideal answer to achieving the aforementioned objectives. The primary goal of this investigation is to develop a sustained release bio-adhesive nano-particulate delivery vehicle for targeted local delivery of gemcitabine for the treatment of pancreatic cancer.

A wide array of synthetic and naturally occurring polymers has been used by different groups to attain a sustained release delivery system. Natural polymers like gelatin, albumin, starch, alginate, chitosan etc are some of the biocompatible and biodegradable compounds approved by FDA for use in pharmaceutical and food industry.

Alginic acid is a water soluble linear polysaccharide extracted from brown algae. The most common species are *Laminaria hyperborean*, *Ascophyllum nodosum* and *Macrocystis pyrifera* [9]. Alginate is also found in some bacteria. It has been isolated from *Azotobacter vinelandii* and some species of *Pseudomonas* [10]. Alginate is commonly used in food industry as a thickener, emulsifier and stabilizer. It has been approved by the FDA as safe upon oral administration. It has been shown to be nontoxic and biodegradable when given orally. However ambiguous results were obtained upon intravenous administration of alginate. Another important quality of alginate that makes it a suitable candidate for use in drug delivery is its bioadhesiveness. It has been proven that polymers with high charge densities exhibit strong mucoadhesive properties, the negative charges on the surface of alginate interact with positively charged mucosal surface leading to strong electrostatic attractive forces. Further studies have provided evidence that alginate has higher

mucoadhesive strength compared to some other bioadhesive polymers like chitosan, carboxymethyl cellulose, poly lactic acid and poly styrene. Alginate has been used for a number of pharmaceutical applications, specifically for controlling the rate of release of therapeutic agents. The delivery systems comprised of alginate include beads, hydro-gels, microspheres, hydrocolloids and nano-particles. Alginate nano-particles formed by multiple emulsion solvent evaporation technique were investigated for entrapment efficiency, in vitro release and cellular uptake of gemcitabine hydrochloride.

Here we introduced some of the research studies have been conducted for optimization of drug delivery system of the gemcitabine as an efficient and purposive cancer treatment modality:

In **Han and coworkers (2017)** study, Dual enzymatic reactions were introduced to fabricate programmed gemcitabine (GEM) nanovectors for targeted pancreatic cancer therapy. Dual-enzyme-sensitive GEM nanovectors were prepared by conjugation of matrix metalloproteinase-9 (MMP-9) detachable poly(ethylene glycol) (PEG), cathepsin B-cleavable GEM, and targeting ligand CycloRGD to CdSe/ZnS quantum dots (QDs). The GEM nanovectors decorated with a PEG corona could avoid nonspecific interactions and exhibit prolonged blood circulation time. After GEM nanovectors were accumulated in tumor tissue by the enhanced permeability and retention (EPR) effect, the PEG corona can be removed by overexpressed MMP-9 in tumor tissue and RGD would be exposed, which was capable of facilitating cellular internalization. Once internalized into pancreatic cancer cells, the elevated lysosomal cathepsin B could further promote the release of GEM. By employing dual enzymatic reactions, the GEM nanovectors could achieve prolonged circulation time while maintaining enhanced cellular internalization and effective drug release. The proposed mechanism of the dual enzymatic reaction-assisted GEM delivery system was fully investigated both in vitro and in vivo. Meanwhile, compared to free GEM, the deamination of GEM nanovectors into inactive 2',2'-difluorodeoxyuridine (dFdU) could be greatly suppressed, while the concentration of the activated form of GEM (gemcitabine triphosphate, dFdCTP) was significantly increased in tumor tissue, thus exhibiting superior tumor inhibition activity with minimal side effects [11].

In this study, researchers looked for ways to increase the effectiveness of gemcitabine by increasing half-life and releasing high doses of drugs in the target cells, and to achieve this, PEGs have been used to produce nanoparticles. In the present study, we seek a common goal through the natural alginate nanoparticle. As with this study, first, the gemcitabine nanoparticle is produced loaded on alginates, then the HPLC method is validated for assessing the drug's effect on the pancreatic cancer cell line and the effect of the new drug combination on increasing the half-life of the drug and the effectiveness of its cumulative dose will be reviewed.

Razzazan and colleagues (2016) have studied the influence of PEG molecular weight on the drug release and in vitro cytotoxicity of single-walled carbon nanotubes-PEG-gemcitabine conjugates. They wrote: Gemcitabine (GEM) is a highly hydrophile anticancer drug which extensively used in the clinic for the treatment of a range of solid tumors, including pancreatic and lung cancers. We have designed a drug delivery system based on single-walled carbon nanotubes (SWCNTs) for the anticancer drug GEM, which has limitations under biological conditions, by using polyethylene glycol (PEG) to obtain nanoconjugates with high loading capacity, controlled drug release and effective cytotoxicity. Raw SWCNTs were functionalized through carboxylation, acylation, PEGylation and finally GEM conjugation via a cleavable ester bond. Different characterization techniques such as Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectrometer (NMR) and differential scanning calorimetry analysis (DSC) were performed to confirm the successful functionalization. Next, the influence of molecular weight (MW) of PEG on the drug loading capacity, drug release and cytotoxicity was studied. Experimental results showed that the drug loading capacity was dependent on the MW of PEG, but the drug release was independent. Also, the results revealed that the nanoconjugates with lower PEG MW caused higher cytotoxicity in A549 and MIA PaCa-2 cancer cells. Their results indicated which of PEG MWs could be useful for this drug delivery system [12].

In this study, the effect of conjugated gemcitabine-containing nanotube has been investigated and PEG has been used to produce nanotubes. In this study, they studied the nanoparticles containing gemcitabine hydrochloride and used natural alginates as a nanoparticles delivery system. Techniques used in this study such as spectrometry and spectrophotometry and calorimetry will similarly be used in the current study.

Sanlier et al (2016) have studied the development of gemcitabine-adsorbed magnetic gelatin nanoparticles for targeted drug delivery in lung cancer. They have coated magnetic iron oxide nanoparticles (IONPs) with gelatin type B by means of the two-step desolvation method. Drug loading by adsorption was studied under various conditions such as different temperature, contact time, pH, and initial gemcitabine concentration. Further, Langmuir isotherm curves were constructed and constants were calculated. According to the Langmuir isotherm, the Gibbs free energy of the adsorption process at 25°C was − 4.74 kJ/mol. On the other hand, this value at 37°C was − 7.86 kJ/mol. In vitro drug release was performed at pH levels of 5 and 7.4, with gemcitabine-loaded magnetic gelatin nanoparticles and free gemcitabine, and both the results were subsequently compared [13].

In the current study, we are also looking for a new targeted drug delivery for the gemcitabine, but unlike Sanlier and coworkers who worked on the lung cancer cell line,

we will study the pancreatic cancer cell line. Moreover, the execution and measurement methods are also validated and expanded, which can be the benefits of the current study.

Şenyiğit and coworkers (2015) studied the design and evaluation of an intravesical delivery system for superficial bladder cancer throughout preparation of gemcitabine HCl-loaded chitosan-thioglycolic acid nanoparticles and they compared chitosan/poloxamer gels as carriers. This study aimed to develop an intravesical delivery system of gemcitabine HCl for superficial bladder cancer in order to provide a controlled release profile, to prolong the residence time, and to avoid drug elimination via urination. For this aim, bioadhesive nanoparticles were prepared with thiolated chitosan (chitosan-thioglycolic acid conjugate) and were dispersed in bioadhesive chitosan gel or in an in-situ gelling poloxamer formulation in order to improve intravesical residence time. They found a 20% improvement in Gemcitabine effects on the bladder cancer cell line. However, when the gel formulations were diluted with artificial urine, poloxamer gels lost their in-situ gelling properties at body temperature, which is in conflict with the aimed formulation property. Therefore, 2% chitosan gel formulation was found to be a more promising carrier system for intravesical administration of nanoparticles [14].

The aim of the present study is increasing the half-life, the timing of metabolism, and the targeted delivery of the gemcitabine, with the exception that alginate natural polymer will be used as the carrier nanoparticle and is tested on the pancreatic cancer cell line.

Recently, a commercial albumin-bound paclitaxel (PTX) nanocarrier (Abraxane) was approved as the first new drug for pancreatic ductal adenocarcinoma in almost a decade. PTX improves the pharmaceutical efficacy of the first-line pancreatic cancer drug, gemcitabine (GEM), through suppression of the tumor stroma and inhibiting the expression of the GEM-inactivating enzyme, cytidine deaminase (CDA). We asked, therefore, whether it was possible to develop a mesoporous silica nanoparticle (MSNP) carrier for pancreatic cancer to co-deliver a synergistic GEM/PTX combination. High drug loading was achieved by a custom-designed coated lipid film technique to encapsulate a calculated dose of GEM (40 wt %) by using a supported lipid bilayer (LB). The uniform coating of the 65 nm nanoparticles by a lipid membrane allowed incorporation of a sublethal amount of hydrophobic PTX, which could be co-delivered with GEM in pancreatic cells and tumors. We demonstrate that ratiometric PTX incorporation and delivery by our LB-MSNP could suppress CDA expression, contemporaneous with induction of oxidative stress as the operating principle for PTX synergy. To demonstrate the in vivo efficacy, mice carrying subcutaneous PANC-1 xenografts received intravenous (IV) injection of PTX/GEM-loaded LB-MSNP. Drug co-delivery provided more effective tumor shrinkage than GEM-loaded LB-MSNP, free GEM, or free GEM plus Abraxane. Comparable tumor shrinkage required co-administration of 12 times the amount of free Abraxane. High-performance liquid

chromatography analysis of tumor-associated GEM metabolites confirmed that, compared to free GEM, MSNP co-delivery increased the phosphorylated DNA-interactive GEM metabolite 13-fold and decreased the inactivated and deaminated metabolite 4-fold. IV injection of MSNP-delivered PTX/GEM in a PANC-1 orthotopic model effectively inhibited primary tumor growth and eliminated metastatic foci. The enhanced in vivo efficacy of the dual delivery carrier could be achieved with no evidence of local or systemic toxicity. In summary, we demonstrate the development of an effective LB-MSNP nanocarrier for synergistic PTX/GEM delivery in pancreatic cancer [15].

Razmimanesh and colleagues (2015) have conducted a molecular dynamics simulation study of chitosan and gemcitabine as a drug delivery system. They've used molecular dynamics (MD) simulation for investigation of biodegradable biopolymer chitosan as a carrier for the drug gemcitabine and the effect of three initial drug concentrations (10, 40, and 80%) on its loading efficiency. Then water was added to the systems of drug and biopolymer and the effects of water on the interactions of drug and chitosan and on the drug loading efficiency were examined. From the results, it was found that the maximum loading of the drug occurred at 40% of the drug concentration. The radial distribution function calculations indicated that in the absence of water molecules, the drug molecules were located at shorter distance from chitosan and the loading efficiency of the drug in these systems was higher.

Razmimanesh and colleagues studied the chitosan polymer, and in this study, we will use the alginate polymer. The suitability and the efficiency of the drug delivery system will be examined. In the Razmimanesh's study HPLC has been used as a survey tool and can be used as the reference to confirm the logic of application of this method in the study of the alginate-gemcitabine drug delivery system in the current study.

Tan and colleagues (2014) have studied the delivering curcumin and gemcitabine in one nanoparticle platform for colon cancer therapy. They've argued as gemcitabine and curcumin have different targets in colon cancer cells, combination of them may bring benefits. Here, curcumin and gemcitabine were formulated into a biodegradable polymer platform for combination therapy for treatment of colon cancer. In doing so, a FDA approved biodegradable polymer mPEG-PLA (methoxyl-polyethylen glycol-block-polylactide) was chosen as a drug carrier. At first, a mPEG-PLA/Gem conjugate was designed. Thereafter, simply using this drug conjugate to encapsulate curcumin, polymeric micelles loaded with both curcumin and gemcitabine were obtained. Varying the feed ratio of the two drugs, a series of micelles with different ratios of curcumin and gemcitabine could be prepared. The as-prepared dual drug loaded nanoparticles showed spherical structures with mean diameters ranging from 118 nm to 149 nm by DLS. In vitro, M(Cur/Gem) almost showed greater synergy than free combination of

curcumin/gemcitabine. In vivo, better antitumor effect and lower systemic toxicity of M(Cur/Gem) were observed on a murine xenograft model. The present study provides the possibility of combining curcumin and gemcitabine in a nanoparticle formulation, and translation of this combination may bring benefits for future clinical use [17].

This study will use gemcitabine as the first line treatment for pancreatic cancer. Moreover, the alginate will be used as the polymer. But the process of nanoparticle preparation and laboratory examination is the same and applied on the same cell line, and the goal is targeted delivery of the drug to cancer cells and increase the efficacy of the drug loading.

Yoshida and co-workers (2012) in a study aimed to targeting anticancer drug delivery to pancreatic cancer cells using a fucose-bound nanoparticle approach, wrote: Owing to its aggressiveness and the lack of effective therapies, pancreatic ductal adenocarcinoma has a dismal prognosis. New strategies to improve treatment and survival are therefore urgently required. Numerous fucosylated antigens in sera serve as tumor markers for cancer detection and evaluation of treatment efficacy. Increased expression of fucosyltransferases has also been reported for pancreatic cancer. These enzymes accelerate malignant transformation through fucosylation of sialylated precursors, suggesting a crucial requirement for fucose by pancreatic cancer cells. With this in mind, we developed fucose-bound nanoparticles as vehicles for delivery of anticancer drugs specifically to cancer cells. L-fucose-bound liposomes containing Cy5.5 or Cisplatin were effectively delivered into CA19-9 expressing pancreatic cancer cells. Excess L-fucose decreased the efficiency of Cy5.5 introduction by L-fucose-bound liposomes, suggesting L-fucose-receptor-mediated delivery. Intravenously injected L-fucose-bound liposomes carrying Cisplatin were successfully delivered to pancreatic cancer cells, mediating efficient tumor growth inhibition as well as prolonging survival in mouse xenograft models. This modality represents a new strategy for pancreatic cancer cell-targeting therapy [18].

They have been developing a special drug delivery system for cisplatin, and reiterated the need to produce and develop new methods for targeted delivery of the pancreatic anticancer drugs. The framework of this study is similar to the framework of our study, and we are also looking for the development of a targeted drug delivery system but for gemcitabine as the first line treatment for pancreatic cancer treatment.

Arsawang and co-workers (2011) pursued the question of how do carbon nanotubes serve as carriers for gemcitabine transport in a drug delivery system? Aiming at understanding the molecular properties of the encapsulation of the anticancer drug gemcitabine in the single-walled carbon nanotube (SWCNT), molecular dynamics (MD) simulations were applied to the two scenarios; that of gemcitabine filling inside the

SWCNT, and that of the drug in the free state. They found that gemcitabine inside the SWCNT have a lower number of solvating water molecules but with a stronger net solvation than the drug in the free state. This is due to the collaborative interactions between gemcitabine and the surface of the SWCNT. In addition, the steered molecular dynamics simulation (SMD) approach was employed to investigate the binding free energy for gemcitabine moving from one end to another end throughout the SWCNT. In excellent agreement with that yielded from the classical MD, the SMD energy profile confirms that the drug molecule prefers to locate inside the SWCNT [19].

In this study, they also sought a way to reduce the time of metabolism and targeted delivery of the gemcitabine that is consistent with the present study. In the present study, one of the main goals is optimization of gemcitabine nanoparticles in order to reduce the hydrophile properties of the compound and increase its half-life, which is consistent with the study of Arsawang and co-workers.

Trickler and colleagues (2010) have studied the chitosan and glyceryl monooleate nanostructures containing gemcitabine. The objectives of this study are to enhance cellular accumulation of gemcitabine with chitosan/glyceryl monooleate (GMO) nanostructures, and to provide significant increase in cell death of human pancreatic cancer cells in vitro. The delivery system was prepared by a multiple emulsion solvent evaporation method. The nanostructure topography, size, and surface charge were determined by atomic force microscopy (AFM), and a zetameter. The cellular accumulation, cellular internalization and cytotoxicity of the nanostructures were evaluated by HPLC, confocal microscopy, or MTT assay in Mia PaCa-2 and BxPC-3 cells. The delivery system demonstrated a significant decrease in the IC50 (3 to 4 log unit shift) in cell survival for gemcitabine nanostructures at 72 and 96 h post-treatment when compared with a solution of gemcitabine alone. The nanostructure reported here can be resuspended in an aqueous medium that demonstrate increased effective treatment compared with gemcitabine treatment alone in an in vitro model of human pancreatic cancer. The drug delivery system demonstrates capability to entrap both hydrophilic and hydrophobic compounds to potentially provide an effective treatment option in human pancreatic cancer [20].

Our methodology will be similar to that of Trickler and colleagues. They've also seeks to create a targeted drug delivery system for increasing the half-life and metabolism time of the gemcitabine. The difference between the present study and theirs is that we use alginate instead of glycerol monovallet to produce nano-structure drug delivery system.

Reddy and Couvreur (2008) in a review study have discussed the strategies adopted to improve the delivery of gemcitabine to tumors. Concomitant research in this area has implemented a wide variety of approaches such as, aerosolized formulations, prodrug

conjugates, liposomes, nanoparticles and beads. Some of these strategies were also aimed at overcoming the rapid metabolization and drug resistance associated with gemcitabine. Aerosolized formulations were employed to treat the local tumors, while other approaches were aimed at the systemic therapy of cancers. The liposomal formulations considerably increased the half-life and the area under the curve (AUC) of gemcitabine, and simultaneously caused a marked improvement in the anticancer activity against experimental solid tumors developed orthotopically or at subcutaneous site. Alternatively, the prodrug conjugates of gemcitabine displayed considerable activity in vivo against various tumors. Especially, in the case of leukemia in which gemcitabine was demonstrated to be inactive, the lipidic conjugates displayed marked efficiency following systemic and oral administration. These conjugates induced greater apoptosis and also caused resistance reversal in the resistant leukemia types. Altogether, the delivery strategies adopted for gemcitabine led to a considerable improvement in the treatment of cancers at the preclinical stage, and some of them are potential candidates for clinical trials [21].

In this study, new strategies for delivery of the gemcitabine to cancer cells have been investigated. One of these strategies is the production of nanoparticles that can be useful in prolonging half-life and metabolism of gemcitabine, but requires further examination and clinical trial. The results of this review study indicate that study of the new methods of drug delivery is necessary and it is a confirmation for the necessity and applicability of the results of the present study.

Research hypothesis

Hypothesis 1: Gemcitabine hydrochloride can be successfully equipped with a sustained release nanoparticle drug delivery system using alginate cross-linking with calcium ions.

Hypothesis 2: The sustained release nanoparticle formulation of gemcitabine hydrochloride can increase the amount of drug taken by the BxPC-3 cell line.

Objectives

To test the above hypotheses, the present study seeks to achieve the following goals:

Objective 1: Development and characterization of a HPLC method for the investigation of gemcitabine hydrochloride

Objective 2: Formulation of a sustained-release drug delivery system containing gemcitabine hydrochloride using alginate cross-link with calcium ions

Objective 3: Evaluation of the gemcitabine hydrochloride uptake from drug delivery system by the BxPC-3 cell line as research model

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Development and Characterization of Polymeric Nanoparticulate Delivery System for Gemcitabine

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