The Investigation of P7 Peptide Delivery Targeting Cdc24 in Ras-driven Pancreatic Cancer by L-fucose-bound Liposome

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Abstract: Pancreatic adenocarcinoma is one of the most common leading causes of cancer deaths with an increasing incidence in the developed world. Most pancreatic cancers are induced by Ras protein abnormality. Since Ras is involved in many important cellular functions, targeting Ras alone is difficult with little progression. Currently, the only surgical resection offers the potential cure to this disease. However, among those who have the chance to receive surgery, most of them suffer from recurrence within a year. The previous study has developed novel peptides targeting Cell division control protein 42 (Cdc42), which is a type of Rho family small GTPase activated by Ras and achieved great success in inhibition of tumor cell growth. Since Cdc42 is also expressed in normal cells, a suitable drug delivery system is required for targeted therapy. This study investigates the cytotoxic and cell penetration effects of L-fucose-bound liposome-P7 targeting pancreatic cancer cells, in both in vitro and in vivo conditions. The experiments will use know human pancreatic cancer cell lines, human pancreatic epithelial cell lines, and Xenograft Murine Models. The concentration of P7 in each L-fucose-bound liposome will be assessed through half-log dilution. Cell proliferation and cytotoxicity are measured through colony formation assay, MTT assay, and Annexin V/ propidium iodide (PI) assay. The cell penetration effect will be reflected by fluorescence microscopy. There are three most possible results: (1) L-fucose-bound liposome-P7 inhibits the pancreatic cancer cell proliferation in both in vitro and in vivo cell lines without causing significant cytotoxicity to normal cells; (2) L-fucose-bound liposome-P7 only inhibits the cancer cell proliferation in vitro cell cultures without causing significant damage to normal cells; (3) L-fucose-bound liposome-P7 inhibits tumor cell proliferation in both normal pancreatic epithelial cells and cancer cells. The result of our study will provide important information for deciding whether to continue P7 peptide development in clinical trials. Future studies should focus on improving the drug delivery system and investigating P7 effects on transformed tumor cells.

1 INTRODUCTION

Pancreatic adenocarcinoma, ranked as the fourth leading cause of cancer deaths in the United States, has poor outcomes and an increasing incidence in the developed world (McGuigan, et al, 2018, Patra, et al, 2010). The incidence rates vary between countries. Generally, the highest incidence rates are detected in Europe and North America, while the lowest incidence rates are measured in Africa and South-central Asia (McGuigan, et al, 2018). This type of cancer often has a late detection and ineffective treatment at the advanced stage, which contributes to a poor 5-year survival rate of 2-9% (McGuigan, et al, 2018). Nowadays, even with advancements in detection and management, the 5-year survival rate remains relatively unchanged with negligible improvements. Currently, the only treatment that offers a potential cure for pancreatic cancer is surgical resection. However, due to the late detection of the disease, only 20% of patients have the chance to receive surgery, and most of them may suffer from disease recurrence within a year (Zeng, et al, 2019). The addition of adjuvant chemotherapy has been shown to improve long-term prognosis in some patients, while other patients have developed chemo-

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resistance with unclear mechanisms, which significantly limits the effect of these therapeutic drugs. Therefore, novel drug developments are necessary (Zeng, et al, 2019).

About 95% of Pancreatic cancer are driven by the mutations in Ras family genes (McGuigan, et al, 2018, Patra, et al, 2010). However, targeting *Ras* gene alone is a hard task since it is involved in many essential signaling pathways promoting cell proliferation and differentiation (Arias-Romero, Chernoff, 2013). Alternatively, targeting the downstream small GTPase that is activated by Ras is favorable. Cdc24 is an important downstream effector of Ras and plays an important role in Ras-induced transformation, and previous studies have developed a novel cyclic peptide, P7, targeting Cdc24 with nanomolar affinity. P7 is tested for binding to the binding surface of Cdc24, preventing it from interacting with its downstream effectors and thus inhibits the transformation pathway in Ras-induced tumorigenesis (Tetley, Murphy, Bonetto et al, 2020). Although the study showed a promising effect of P7 peptide as a Cdc24 inhibitor in Ras-driven cancers, the peptide entry strategy has become another challenge. Cell-penetrating peptide (CPP) was tagged to the P7 and helped the delivery in the previous study. However, the results showed a significant cytotoxic effect with reduced target engagement (Tetley, Murphy, Bonetto et al, 2020). Therefore, an alternative drug delivery system with minimal cytotoxicity and greater efficacy is necessary to be developed.

Nanoscale drug delivery system using liposomes is an emerging technology in cancer treatment. Liposomes are composed of a lipid bilayer that is enclosed as a hollow sphere with an aqueous phase inside. Accordingly, it can encapsulate and stabilize drugs in either aqueous compartments or lipid bilayers, depending on the properties of drugs. Also, the similarity of liposomes to the biological membrane reduces their toxicity and enables the enhanced permeability and retention (EPR) effects to tumor tissues (Malam, Loizidou, Seifalian, 2009). Specifically, most solid tumors have the nature of vascular abnormalities, like hypervascularization, aberrant vascular architecture, and a lack of lymphatic drainage (Malam, Loizidou, Seifalian, 2009). Taking advantage of the adjustability of nanoparticles' size, nanoscale anticancer drugs designed ideally in a moderate size are unable to penetrate through tight endothelial junctions of normal blood vessels. However, they can selectively extravasate in the tumor tissue relying on the tumor's abnormal vascular characteristics, thereby reaching several fold drug concentrations in the tumor than that in the normal tissue (Malam, Loizidou, Seifalian, 2009). Therefore, liposome is a good candidate for P7 peptide delivery.

The surface of liposomes can be modified by taking advantage of characteristics of pancreatic adenocarcinoma. Since 80% of pancreatic cancer cells overexpress carbohydrate antigen-19-9 (CA19-9) and thus recruit large amounts of fucose as an energy source, the fucose-bound liposome can be generated for targeted delivery (Papahadjopoulos, Heath, Bragman, Matthay, 1985, Yoshida, Takimoto, Murase, et al, 2012). A previous study has applied a 14C-labeled L-fucose binding assay in the pancreatic cell lines, and the result indicated the presence of high-affinity L-fucose specific receptors (Yoshida, Takimoto, Murase, et al, 2012). Furthermore, the inhibition of endocytosis by chroloquine resulted in a suppression of drug delivery (Yoshida, Takimoto, Murase, et al, 2012). These results together supported that L-fucose-bound liposome enters the pancreatic cancer cells via receptor-mediated endocytosis (Yoshida, Takimoto, Murase, et al, 2012).

In order to investigate the drug delivery system with minimal side effects and better efficacy, a comparative study should be designed. In the present study, we asked whether targeting the delivery of P7 with L-fucose-bound liposome can increase the cell penetration, enhance cytotoxicity to tumor cells and reduce cytotoxicity to normal cells compared with CPP-tagged P7 both in *in vitro* and *in vivo* conditions. We chose BxPC-3 and AsPC-1 pancreatic cancer cell lines, which secreted substantial amounts of CA-19-9 molecules (Yoshida, Takimoto, Murase, et al, 2012). We hypothesized that treatment of P7 peptide delivered in L-fucose liposomes to Ras-driven pancreatic cancer can increase cell penetration, peptide stability and reduce cytotoxicity to normal cells. In the present study, we will treat AsPc-1, BxPC-3, and normal pancreatic epithelial cells (hTRET-HPNE) in culture or as a mouse xenograft model with increasing amounts of P7-liposome assessed by half log dilution series with various liposome and/or P7 peptide concentrations and measure cytotoxicity by cell counts, colony formation assay, MTT assay, Annexin V/PI, and cell penetration by fluorescently labeled liposomes.

2 METHOD & MATERIAL

This experiment will use two human pancreatic cancer cell lines (AsPC-1 and BxPC-3), and one well studied non-cancerous pancreatic cell line (hTRET-

HPNE) for in vitro studies and mouse xenograft models for in vivo studies. Mouse xenograft models include new subcutaneous and orthotopic models which are generated from previous research and will be used for the *in vivo* study (Yoshida, Takimoto, Murase, et al, 2012). The subcutaneous model will establish with mice bearing AsPC-1 cell lines, which the orthotopic model will use mice bearing BxPC-3 cell lines. The mice will be housed under specific pathogen-free conditions. Animal studies will be carried out according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All surgery will be performed under sodium pentobarbital anesthesia, and all efforts will be made to minimize suffering. P7 peptide and L-fucose bound liposomes will be made according to the method used by Tetley et al (Tetley, Murphy, Bonetto et al, 2020). and Yoshida et al (Yoshida, Takimoto, Murase, et al, 2012).

2.1 Material

PBS (pH 7.4), Purified P7 peptide, L-fucose-bound liposome-P7, Crystal violet 0.5% (wt/vol) in H2O, Glutaraldehyde 6.0% (vol/vol), Trypsin-EDTA (0.05%), Appropriate culture medium containing serum, RPMI 1640 (Gibco) plus 10% FBS, L-glutamine, 1% penicillin-streptomycin, and binding buffer 10X: 0.1 M HEPES/NaOH, pH 7.4; 1.4 M NaCl; 2.5 nM CaCl, Class 2B biocabinet, 500 mg MTT powder, methanol, ethanol, DMS and acidified isopropanol, Plateshaker, Pipettes 0.001–1 mL, single channel and 0.01–0.3, multichannel, Class 2B hood, Benchtop centrifuge, Microplate reader, O2 incubator, L-fucose-bound liposome-P7, L-fucose-bound liposome without P7, liposome alone, CPP-P7, P7 alone and PBS.

2.2 Method

a) Half log dilution

To find out the optimal drug concentration of P7 and L-fucose-P7 liposomes, half-log dilution will be applied in both in vitro and in vivo studies. The stock solution of pure P7 without liposome will be prepared by dissolving P7 peptide in PBS (pH=7.4) in a concentration of 1mg/ml. Varying concentrations of P7 solutions will be constructed by diluting the stock solution with PBS to seven final concentrations at 0.1mg/ml, 0.0316mg/ml, 10 ug/ml, 3.16 ug/ml, 1 ug/ml, 0.316 ug/ml, 0.1 ug/ml. These solutions will be used to test their cytotoxicity and proliferation inhibition effect on cell lines. Similarly, the stock solution of L-fucose-bound liposome-P7, keep the amount of P7 inside the liposome the same as the pure P7 group.

Then, L-fucose-bound liposome-P7 stock solution will be parallelly diluted with P7 group, resulting in varying concentrations with the inside p7 concentrations of 0.1mg/ml, 0.0316mg/ml, 10 ug/ml, 3.16 ug/ml, 1 ug/ml, 0.316 ug/ml, 0.1 ug/ml, which will be used to test cytotoxicity and proliferation inhibition effect on cell lines. The concentration range of P7 and L-fucose-bound liposome-P7 concentration that achieve the significant inhibitory effect will be obtained from the in vitro MTT assay. Subsequently, three doses will be selected to test in vivo studies for testing treatment effects, determining the optimal in vivo drug concentration, which will be used in subsequent experiments.

b) Peptide synthesis and purification

Table 1: The peptide sequence of P7 (Tetley, Murphy, Bonetto et al. 2020).

Peptide	Sequence															
P7	P	S	I	C	H	V	H	R	P	D	W	P	C	W	Y	R
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Table 1 shows the sequence of P7 peptide. Synthesis and purification of the target peptide use the method from Tetley et al (Tetley, Murphy, Bonetto et al, 2020). Standard Fmoc solid-phase chemistry is used to synthesize a 10μmol scale peptide with an amidated C terminus on the automated peptide synthesizer. After the deprotection of Fmoc, the N terminus will be acetylated and biotinylated as described in Tetley et al (Tetley, Murphy, Bonetto et al, 2020). Subsequently, the resin is washed with methanol and dichloromethane, then dried overnight. 5% water, 5% phenol, 5% thioanisole, 2.5% ethanedithiol in TFA is used to deprotect and cleave the peptide from the resin for 3 h. Then, the peptide will precipitate in diethyl ether at -20 °C. A C18 column in reverse-phase HPLC is used

to purify the peptide with a linear gradient elution of 20 to 50% acetonitrile in 0.1% TFA. Then, the determination of molecular masses uses a mass spectrometry.

c) Preparation of CPP-tagged P7

The addition of CPP, which is a C-terminal Nona-arginine motif (9R), is the same as the motif used in Tetley et al (Tetley, Murphy, Bonetto et al. 2020). Carboxyfluorescein (FAM) will be linked to the N-terminal of the P7 peptide. Named the product without FAM and with FAM as CPP-tagged-P7 and CPP-tagged-P7-FAM, respectively.

d) Preparation of P7 encapsulated in liposomes

Preparation of L-fucose bound liposomes has been described previously (Tetley, Murphy, Bonetto et al. 2020). Generally, DPPC, Chol, ganglioside, DCP, and DPPE will be mixed at different molar ratios, and cholic acid will be added for micelle formation. The mixture will be dissolved in methanol/chloroform (1:1, v/v), and the solvent will be evaporated at 37°C to produce a lipid film, which will be dried under vacuum. For the P7 peptide preparation, P7 peptide containing solution will be added to the lipid film and sonicated to obtain uniform micelles in the buffer, which will then be ultrafiltered. Hydrophilization treatment and L-fucose conjugation on the surface of liposomes will be carried out by methods modified from Yamazaki et al (Yamazaki, Kodama, Gabius, 1994). Aminated L-fucose will be conjugated to the liposome surface using DTSSP, which is a type of cross-linking agent.

e) In vitro cell culture

The pancreatic cell lines AsPC-1 and BxPC-3 will be cultured in RPMI 1640 (Gibco) plus 10% FBS, L-glutamine, and 1% penicillin-streptomycin (Yamazaki, Kodama, Gabius 1994). Non-cancerous pancreatic cell line (hTRET-HPNE) will be cultured in DMEM (Gibco) with 10% FBS, 5% L-glutamine, and 1% penicillin-streptomycin. All cell lines will be kept in a 5% CO2 humidified atmosphere at 37 °C.

f) Colony formation assay

In our study, we use cell colony formation assay to measure the ability of a cell to divide and form a colony after delivering P7 peptide delivered in l-fucose liposomes to Ras-driven pancreatic cancer. We will first prepare 6-well plates and harvest exponentially growing cells. Then, we replate 50 cells per dish, waiting for 2 h at 37 Degree Celsius. Followed by incubation in the incubator until cells in control dishes have formed sufficiently large clones. Harvest cells from a donor culture using trypsinization, which makes the cells completely detach from the flask and float in the medium, allowing us to see the cells more clearly. When the cells start to detach from the culture dishes, resuspend the cells in medium to inhibit trypsinization. Then, neutralize the trypsin solution and count the cells. Dilute the cell suspension and seed into flasks. Remove the medium and rinse the cell by PBS. After PBS wash, 2-3 ml of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet will be added. Leave this for at least 30 min. Finally, remove the glutaraldehyde crystal violet mixture and dry at room temperature. After this, we count the colony formation of cells in different plates that with the different treatments. We will use Equal. 1 to calculate the surviving fraction of cells.

$$\mathbf{PE} = \frac{\textbf{Number of colonies counted}}{\textbf{Number of cells plated}} \times \mathbf{100} \qquad \text{Equal. 1}$$

(Franken, Rodermond, Stap, Haveman, Van-Bree 2006)

g) MTT assay

In this study, we will use MTT assay to investigate the killing effect of targeted drugs (L-fucose-bound liposome-P7) on pancreatic cancer cells and drug sensitivity in established cell lines. Subsequently, MTT assay is also used to determine drug concentration that is required to achieve 50% growth inhibition as compared to the growth of the untreated control (50% inhibitory concentration, IC50). Briefly, set up bottom plates (120 mL) to be filled with PBS. If the drug is unstable, the drug diluent will be freshly prepared before adding cells, and 20 ml of total drug and 30 ml of drug solution must be added to the plate. If testing a stable drug, plates can be prepared with 30 mL drug concentrations and can be stored at −20°C for later use. Cultured pancreatic cell lines for 4 days to determine the optimal effect for most standard drugs. After the appropriate incubation time, add 1:10 volume of MTT solution (5 mg/mL), Shake plates for 5 min on a plate shaker by slowly increasing the shaking speed to a maximum of 900 shakes/min, then incubate the plate for another 4–6 h at 37°C in a CO_2 incubator. 150 mL of acidified isopropanol is added to each well, mixing the rows with drugs. The cell lines were divided into six groups and mixed with drugs according to the table. 150 mL of acidified isopropanol is added to each well, mixing the rows with drugs. The cell lines were divided into six groups and mixed with drugs according to table 1. In terms of measurement, the optimal density (OD) is measured at 540 and 720 nm to get a more exact measurement. The pancreatic cell survival is calculated by: (OD treated well [−blank])/(mean OD control well [−blank])×100. The LC50 (the drug concentration which results in 50% pancreatic cell survival) can be calculated.

Table 2: Different drugs put in the cell lines (Van-Meerloo, Kaspers, Cloos 2011).

	AsPC-1 cell line			BxPC-3 cell line		
Serial number	1	2	3	4	5	6
L-fucose-bound liposome	-	+	+	-	+	+
P7	-	-	+	-	-	+

Note. "+" represents a significant decrease in cell proliferation compared with negative controls*. "-" is not significantly different from negative control.

Table 2 shows the possible results of cell proliferation of L-fucose-liposome and P7 peptide in two different cell lines (AsPC-1 and BxPC-3) in MTT assay.

h) Fluorescence polarization

Cell penetration effect will be detected by confocal laser microscopy and fluorescence microscopy. As discussed in previous work (Tetley, Murphy, Bonetto et al, 2020), cells will be plated in Lab-Tek chambered coverglasses at 1 X 10 ^{4} cells/chamber (liposome citation). L-fucose-liposome-P7 or liposome-P7 will be added to cells at an optimal P7 concentration determined by half log dilution (predicted around 0.7μg/ml). There will be six different groups. The experimental group includes FAM-L-fucose-P7 liposome. The negative control group includes FAM-P7, FAM-liposome, FAM-L-fucose liposome, and FAM-L-fucose. Positive control group consists of FAM-CPP-P7 alone. Each treatment will be mixed with cancer cell lines and normal cells. Then each group will receive a beam of Fluorescence microscope which will be used to detect the intensity of excited fluorescent probes (Moerke 2009).

If L-fucose-p7 liposome is not bound to the Cdc42 protein, when they are hit by polarized light, the light is going to be deflected. Since the complex is small, the angle of rotation will be big, and the light it emits is unpolarized, which is more diffuse and less intense, so the degree of deflection will not be strong (Moerke 2009). However, when the L-fucose-P7 liposome binds to the Cdc42 protein, the complex becomes more massive. The Cdc42 protein is too large to rotate freely, so the large complex keeps the light in the polarized state, hence producing very fluorescent light. By comparing the fluorescence intensity, whether the Cdc42 protein binds to the drug can be known. If the drug binds to Cdc42 protein, very strong fluorescence can be obtained. On the contrary, if they are not combined, the fluorescence intensity will be weak. By comparing the content of fluorescent agents in cells, the cell penetration of drugs can be known. For example, the higher the intensity of fluorescent light in the cell, the higher the amount of drug entering the cell, so the cell is more penetrating. However, if the content of fluorescent agents in cells is relatively low, it indicates that the drug content of forbidden cells is relatively low, and the cell penetration is poor.

i) Annexin V/PI assay

In order to assess the cytotoxicity of newly synthesized L-fucose-liposome-P7 compared with previously developed CPP-P7, we apply annexin V/PI assay to determine the fraction of apoptotic and necrotic cells (Kabakov, Gabai, 2018). We will first harvest the AsPC-1, BxPC-3 and hTRET-HPNE cell lines and wash twice in PBS at 4 degree celsius and resuspend in 1 X buffer. Then aliquot 100 µl cells into fluorescence-activated cell sorter (FACS) tubes and add 5 µl FITC-Annexin V and/or 10 µl PI (50 ug/ml Propidium Iodide 10x). The mixture will incubate for 15 min at room temperature in the dark. Finally, 400 µl binding buffer (Binding buffer 10x : 0.1 M HEPES/NaOH, pH 7.4; 1.4 M NaCl; 2.5 nM CaCl) will be added to each tube and the result will be analyzed by flow cytometry on FACS.

j) Fluorescence microscopy

Cell penetration effect will be detected by fluorescence microscopy. As discussed in previous work, cells will be plated in Lab-Tek chambered cover glasses at 1 X 10 ^{4} cells/chamber (Tetley, Murphy, Bonetto et al. 2020). L-fucose-liposome-P7-GFP or liposome-P7-GFP will be added to cells at an optimal P7 concentration determined by half log dilution (predicted around 0.7μg/ml). According to Tetley et al. (Tetley, Murphy, Bonetto et al, 2020), cells will be cultured in complete medium for 30 min and then replaced with fresh medium. Cells will be washed twice with PBS and fixed with 4% paraformaldehyde 30 min and 2 hours post-treatment at room temperature for 15 min. Then, the cells will be washed 3 times using PBS and exposed to DAPI staining the nuclei. The distribution of P7 peptide can

be assessed by fluorescence microscopy by comparing the fluorescent intensity.

k) Peptide stability study

Inject P7 and L-fucose-bound liposome-P7 into mice from the subcutaneous model group and detect the drug plasma concentration after injection of 1, 2, 4, 8, 12 and 48 hours. Make profiles of drug plasma concentration with time by HPLC. Compare the drug plasma concentration curve, the stability of peptide to the serum protease can be analyzed.

l) Animal model development

The subcutaneous model and orthotopic model have been previously described (Tetley, Murphy, Bonetto et al. 2020). Briefly, in the subcutaneous model, mice aged 4 to 6 weeks will be modified with AsPC-1 cell line to allow the growth of tumor to 5 mm in diameter. AsPC-1-bearing mice will be treated with CPP-tagged P7 (2 mg/kg), L-fucose-P7-Liposome (2 mg/kg), via the tail vein twice a week. At 4, 8, 11, 15, 18, and 22 days after transplantation, tumor volumes will be measured by IVIS imaging. Representative image of mice treated with P7. For D-mannose pre-treatment in the *in vivo* experiment for L-fucose bound liposome, 5 mg of D-mannose will be injected through the tail vein 5 minutes before administration of agents (Tetley, Murphy, Bonetto et al, 2020).

In the orthotopic model, BxPC-3-Luc cells in 100 μl PBS will be orthotopically injected into the pancreas of nude mice (ages 4 to 6 weeks). Bioluminescence will be measured on day 0-4 post-injection, and the mice will be randomly assigned into different groups (placebo groups, CPP-tagged P7 group and L-fucose bound liposome-P7 group) before the initiation of treatment. The mice received injection twice in the first week, and then received injection once in week 2 and once in week 3. All mice will be sacrificed on the day after the last injection but before a final bioluminescence measurement.

m) Statistical Analysis

Results will be presented as means (± SD) for each sample. All statistical significance of all numerical data will be analyzed using the student's T-Test on GraphPad Prism® at ($p < 0.05$).

3 POSSIBLE RESULTS

Table 3: In Vitro Cell proliferation comparison of L-fucose-liposome-P7 with negative controls on cancer cells and normal cells.

	Result 1		Result 2		Result 3		Result 4		Result 5	
	C	N	C	N	C	N	C	N	C	N
Colony formation assay	+	-	+	_	++	+	+	+	-	-
MTT assay	+	-	+	-	++	+	+	+	-	-
Annexin /PI assay	A	L	A	L	D	D	A	A	L	L

Note. In table 3, "C" represents tests in cancer cells group and "N" means in normal cells. "+" represents a significant decrease in cell proliferation compared with negative controls*. "-" is not significantly different from negative controls. Negative controls include L-fucose-liposome, Liposome without L-fucose and L-fucose. "A" means apoptotic cell population is significantly larger than live and necrotic cells, while "L" represents live cells population is larger than apoptotic and necrotic cells and "D" represents population of necrosis dead cells is higher than both apoptotic and necrotic cells.

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Table 4: Cell proliferation inhibition comparison of L-fucose-liposome-P7 with P7 and CPP-P7.

	Result 1		Result 2		Result 3		Result 4		Result 5	
	P7	Cpp-P7	P7	Cpp-P7	P7	Cpp-P7	P7	Cpp-P7	P7	Cpp-P7
Colony formation assay	+	++	-	+	++	+	-	+	-	-
MTT assay	+	++	-	+	++	+	-	+	-	-

Note. In table 4, "+" represents a significant higher cell proliferation inhibition effect of L-fucose-liposome-P7 in cancer cell lines compared with that of p7 and cpp-p7, respectively. "-" indicates that no significant greater proliferation inhibition effect of L-fucose-liposome-P7 compared with p7 or cpp-p7, respectively.

Table 5: Possible Results on Cell penetration of L-fucose-liposome-P7 in vitro.

Cell penetration by L-fucose-liposome-P7 in vitro	Result 1	Result 2	Result 3	Result 4	Result 5
Fluorescence microscopy	+	+	+	-	+

Note. In table 5, "+" represents the fluorescence intensity inside the cancer cells is significantly higher than that outside. "-" indicates that fluorescence intensity inside cancer cells is not significantly higher than outside.

3.1 Possible Results

Possible result 1: As shown in table 3 and table 4, L-fucose-bound liposome-P7 has significantly greater cell anti-proliferative effect to pancreatic cancer cells compared with all negative controls, P7 peptide and CPP-P7. Also, fluorescence intensity indicates that L-fucose-bound liposome-P7 has good penetrability to cancer cells (Table 5). Moreover, the sizes of tumors in animal models have significantly decreased by treating with L-fucose-bound liposome-P7. Additionally, L-fucose-bound liposome-P7 shows significant higher inhibitory effects on the cell growth of in vitro cancer cell samples and in vivo xenograft mice models without affecting normal pancreatic epithelial cells.

Possible Result 2: L-fucose-bound liposome-P7 exerts significant greater inhibitory effect on proliferation of the pancreatic cancer cells in the determined human pancreatic cancer cell lines, compared with CPP-P7 and negative controls but not P7 peptide. Also, it shows cytotoxicity to pancreatic cancer cells but not non-cancerous cells, and it controls/reduces mean tumor diameter in vivo animal models without affecting normal pancreatic cells.

Specially, L-fucose-bound liposome-P7 show greater cytotoxicity and inhibitory effect on proliferation of pancreatic cancer cell lines but no significant inhibitory effect and cytotoxicity on normal pancreatic cell lines compared with all negative control groups. Also, L-fucose-liposome, Liposome without L-fucose and L-fucose alone do not show inhibitory effect on growth of both pancreatic cancer cell lines and normal pancreatic cell lines, while P7 alone shows comparable cell proliferation inhibition to L-fucose-bound liposome-P7 on pancreatic cancer cell lines.

Possible Result 3: L-fucose-bound liposome-P7 shows cytotoxicity to cancer cells to a large extent and revels cytotoxicity to normal cells (Table 3). Also, L-fucose-bound liposome-P7 reduces mean tumor diameter in vivo mice models but causes side effects to mice.

L-fucose-bound liposome-P7 exerts greater cytotoxicity to both pancreatic cells and normal pancreatic epithelial cells in vitro and in vivo. Also, all negative control groups do not show significant cell cytotoxicity to the pancreatic cancer cells in vitro and in vivo studies. As shown in table 3, results of Annexin V/PI assay indicates that L-fucose-bound liposome-P7 causes cell deaths by necrosis to pancreatic cells and normal pancreatic epithelial cells. In table 4, L-fucose-bound liposome-P7 shows a significant higher cell proliferation inhibition effect in cancer cell lines compared with P7 and CPP-P7. Moreover, fluorescence microscopy results show that

L-fucose-bound liposome-P7 has poor penetration to cancer cells (Table 5).

Possible Result 4: L-fucose-bound liposome-P7 inhibits cell growth of both pancreatic cancer and normal cells (Table 3). Fluorescence imaging results reflect poor cell penetration of L-fucose-bound liposome-P7 to pancreatic cancer cells.

L-fucose-bound liposome-P7 exerts great inhibitory effects on cell growth of cancer cells and normal cells mainly by apoptosis (Table 3). In vivo animal study, the sizes of tumors are decreased by L-fucose-bound liposome-P7. As shown in table 4, L-fucose-bound liposome-P7 shows a significant higher cytotoxicity to cancer cell lines compared with CPP-P7 but not P7. FAM-L-fucose-liposome and FAM-L-fucose-bound liposome-P7 are observed to locate and accumulate significantly in tumor sites, while FAM-P7, FAM-liposome, FAM-L-fucose and FAM-CPP-P7 do not show significant target delivery to the pancreatic tumor site.

Possible Result 5: L-fucose-bound liposome-P7 does not exert any significant effect to either pancreatic cancer cells, or normal pancreatic epithelial cells (Table 3). Also, all negative control groups show not significant effect on the growth of both pancreatic cancer cells and normal pancreatic cells. Colony formation assay, MTT assay and Annexin assay all demonstrate insignificant effect on pancreatic cancer cells and normal cell lines (Table 3). Although L-fucose-bound liposome-P7 shows good cell penetration to cancer cells (Table 5), it has no comparable cytotoxicity to P7 and CPP-P7 on in vitro pancreatic cancer cells (Table 4). Also, fluorescence imaging experiments of FAM-L-fucose-bound liposome-P7 and all negative control groups including FAM-P7, FAM-liposome, FAM-L-fucose liposome, and FAM-L-fucose and positive control (FAM-CPP-P7) do not show significant distribution and accumulation in the targeted pancreatic tumor site.

4 DISCUSSION

Previous studies report that P7 peptide binds with Cdc42 protein and inhibits the downstream functions exerted by Ras-mediated signaling, leading to blockage of cancer cell transformation in Ras-driven cancers (Malam, Loizidou, Seifalian, 2009). However, the lack of a proper delivery system slows down the drug development processes. To determine a better delivery system that would enhance therapeutic effects of P7 peptide and reduce OFF-target cytotoxicity, this study uses L-fucose-modified liposome in comparison with CPP-tagged P7 peptide used in previous studies to deliver P7 peptide to two well studied pancreatic cancer cell lines from humans and to in vivo pancreatic cancer animal models. Possible result 1 indicates a great cytotoxic effect and proliferation inhibitory effect on pancreatic cancer cells without affecting normal pancreatic epithelial cells, indicating target delivery of L-fucose-bound liposome-P7 to pancreatic cancer cells. Fluorescence imaging indicates that L-fucose-bound liposome-P7 has good penetrability to cancer cells, which may explain its higher cytotoxicity than P7. According to Tetley et al. (Tetley, Murphy, Bonetto et al. 2020), binding with CPP reduces the efficacy of P7, which explain the much higher cytotoxicity of L-fucose-bound liposome-P7 than CPP-P7 (Table 3). Overall, possible result 1 fully supports our hypothesis that L-fucose-modified liposome has a significant treatment effect with enhanced cell penetration and targeted delivery to pancreatic cancer cells. Further studies assessing the pancreatic cancer cell transformation should be done for a thorough understanding of the blockage of Ras-driven transformation. More complex and representative animal models should also be done in preclinical trials before entering clinical trials to prevent potential damages that could not be found in mouse models.

Similar with possible result 1, possible result 2 indicates L-fucose-modified liposome P7 peptides achieve the great efficacy with minimal side effects and toxicity, which is consistent with previous investigations in targeted delivery of L-fucose liposome to pancreatic cancer cells. Differently, in possible result 2, L-fucose-modified liposome P7 shows *significant greater proliferation inhibition effect than cpp-p7 but not p*7, which indicates that the efficacy of P7 is not enhanced by L-fucose liposome. This could be also possibly because binding with L-fucose liposome reduce the efficacy of P7 when increasing drug distribution of L-fucose-bound liposome-P7 to cancer cells. This result partially supports our hypothesis that L-fucose-modified liposome has targeted drug delivery to the tumor site and increased cell penetration to cancer cells.

Compared with possible result 1 and 2, possible result 3 shows that L-fucose-modified liposome P7 peptides has strong cytotoxicity and side effects at the same time. It may be due to the existence of other unknown molecular mechanisms induced by L-fucose-modified liposome P7 peptides in the cell, resulting in the apoptosis of normal cells and pancreatic cancer cells induced. Simultaneously，in result 3 both L-fucose-modified liposome P7 and cpp-p7 shows significant proliferation inhibition and

L-fucose-modified liposome P7 is stronger, which may because the 9R motif binds non-specifically to other moieties or causes aggregation in the lysate(Tetley, Murphy, Bonetto et al, 2020).This result partially supports our hypothesis that L-fucose-modified liposome has targeted drug delivery to the tumor site but also has strong cytotoxicity to the normal cell.

Possible result 4 shows that L-fucose-modified liposome P7 peptide has cytotoxicity to both cancer cells and normal cells by apoptosis (Table 4), while L-fucose-modified liposome P7 has poor penetration to the cancer cells, indicating the possibility that L-fucose-modified liposome P7 induce an unknown path on the cell surface which cause apoptosis of cells. compared with cell. by cell apoptosis, cell intracellular content is not released while by cell necrosis, cell intracellular content is released which is prone to cause inflammatory reaction (Helewski, Kowalczyk-Ziomek, Konecki, 2006). Hence, drug cytotoxicity induced by cell apoptosis is more desirable than by cell necrosis. However, L-fucose-modified liposome P7 causes significant harm to normal pancreatic cells which would potentially increase the suffering of patients. Hence, future work should modify liposome carriers or find a new strategy to increase the cell penetration of P7, which can eventually achieve the efficacy of P7. Overall, possible result 4 does not support our hypothesis.

There could be many reasons for the failure of possible result 5. However, we have set up several negative controls to eliminate the problem. If it does not show any alteration compared with negative controls, which include P7 alone, liposome alone, L-fucose-liposome alone, it is likely that P7 is unable to exert its inhibitory effect in pancreatic cancer cells. If P7 alone shows a significant inhibitory effect in pancreatic cancer cells compared with L-fucose-liposome-P7, then probably the delivery system is the problem that prevents P7 from exerting its effect. Other failed results in animal models could be due to enzymatic activity in the animal body, clearing liposomes before they reach the pancreas. Therefore, further study should investigate liposome modifications that make them last longer in the animal body is necessary.

5 CONCLUSION

In conclusion, the objective of our experiment was to determine whether targeting P7 delivery with L-focused binding liposomes can increase cell penetration, enhance cytotoxicity to tumor cells, and reduce cytotoxicity to normal cells compared to CPP-tagged P7 under in vitro and in vivo conditions. This study explores the therapeutic effect of L-fucose-P7 liposome in CA19-9 overexpressing pancreatic cancer cell lines and Xenograft Murine Models. The results of this study will examine whether L-fucose-P7 liposome has a better therapeutic effect and a weaker OFF-target effect compared with CPP-tagged-P7 used in previous studies and preparing it for entering clinical trials. These possible results and the experiment itself provide the potential for future improvement in the delivery system including adjustment of liposome size to increase half-life and adding monoclonal antibodies specific for pancreatic cancer cells overexpressing EGFRs. The modifications on liposome surface proteins will alter pharmacokinetics of the P7 peptide to make it more efficacious and the enhanced targeting skill of the liposome will potentially minimize the OFF-tumor effects.

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