財團法人明日醫學基金會專題研究計畫申請書

一、基本資料： 申請條碼：

本申請案所需經費(單選)	■A 類(執行計畫所需經費) ☐B 類(研究主持費，限人文處計畫，不須填寫表 C002 及 C004 至 C009)				
計畫類別(單選)	■一般型研究計畫 ☐特約研究計畫 ☐新進人員研究計畫 ☐其他				
研究型別	■個別型計畫 ☐整合型計畫				
申請機構/系所(單位)	長庚大學醫學院微生物及免疫學科				
本計畫主持人姓名	賴志河	職稱	教授	身分證號碼	
本計畫名稱	中文	空腸彎曲桿菌細胞致死腫脹毒素的應用			
	英文	Theranostic application of *Campylobacter jejuni* cytolethal distending toxin			
整合型總計畫名稱					
整合型總計畫主持人				身分證號碼	
全程執行期限	自民國 110 年 1 月 1 日起至民國 110 年 12 月 31 日				
研究學門(請參考本申請書所附之學門專長分類表填寫)	學門代碼	名稱(如為其他類，請自行填寫學門)			
研究性質	■純基礎研究 ☐導向性基礎研究 ☐應用研究 ☐技術發展				
本計畫是否為國際合作計畫	■否； ☐是，合作國家：_____，請加填表 I001～I003				
本計畫是否申請海洋研究船	■否； ☐是，請務必填寫表 C014。				
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三、主要研究人力：

（一）請依照「主持人」、「共同主持人」、「協同研究人員」及「博士後研究」等類別之順序分別填寫。

類 別	姓名	服務機構/系所	職稱	在本研究計畫內擔任之具體工作性質、項目及範圍	*每週平均投入工作時數比率(%)
主持人	賴志河	長庚大學/醫學院/微生物及免疫學科	教授	規劃及推動研究進行、實驗設計、整理數據及撰寫研究成果與論文	70%

※註：每週平均投入工作時數比率係填寫每人每週平均投入本計畫工作時數佔其每週全部工作時間之比率，以百分比表示（例如：50%即表示該研究人員每週投入本計畫研究工作之時數佔其每週全部工時之百分五十）。

Abstract

Campylobacter jejuni cytolethal distending toxin (CDT) is composed of CdtA, CdtB, and CdtC. Amongst, CdtB possesses a deoxyribonuclease I (DNase I) activity that causes double-strand DNA break (DSB) in the nucleus, leading to cell cycle arrest and cell death. We recently demonstrated that CdtB can be employed as a therapeutic agent for the treatment of radio-resistant prostate cancer (PCa) cells. The radio-resistance and increasing cancer stem cells (CSC) are closely associated with the loss of DOC-2/DAB2 interactive protein (DAB2IP). Our previous study reported that CdtB sensitizes radio-resistant PCa to ionizing radiation. This study will further use nanoparticles to encapsulate CdtB that specifically targets to radio-resistant PCa cells. The results from this study will develop a novel theranostic agent for the refractory PCa therapy.

Keywords: nanoparticles; cytolethal distending toxin; prostate cancer; radio-resistance

A. Background introduction

Campylobacter jejuni and cytolethal distending toxin (CDT)

C. jejuni, a Gram-negative bacterium, is a causative agent of food-borne diseases in humans [1]. Infection by *C. jejuni* occurs through the consumption of contaminated poultry products and is typically associated with a local acute inflammatory response that involves intestinal tissue damages [2]. Cytolethal distending toxin (CDT), a virulence factor of *C. jejuni*, is composed of three protein subunits: CdtA, CdtB, and CdtC [3]. Among the three subunits, CdtB is a type-I deoxyribonuclease (DNase I), which causes DNA double-strand break (DSB), cell-cycle arrest at the G2/M phase, leading to cell distention and death [4,5]. Our previous study demonstrated that CDT is associated with cholesterol-rich microdomains of cells in a modality similar to the CDTs from *Haemophilus ducreyi* and *Aggregatibacter actinomycetemcomitans* [6]. We also found that *C. jejuni* CDT induces severe intestinal inflammation in mice fed with a high-cholesterol diet, demonstrating that cholesterol and lipid rafts are crucial in facilitating CDT-induced pathological derangement *in vivo* [7,8].

Refractory cancer therapy by using bacterial toxins

Therapeutic resistance often involves the presence of a major molecular efflux pump in cell membranes, enabling cancer cells to bypass drug toxicity or alter cellular processes between the cytoplasm and the nucleus [9]. It may be beneficial to use bacterial toxins as alternatives to treat refractory tumors, given that these toxins are easily obtainable from bacteria and the toxins can efficiently gain access to target cells through membrane-receptor internalization. Several bacterial toxins have been applied in clinical settings for cancer therapy, including the anthrax toxin from *Bacillus anthracis* [10], diphtheria toxin from *Corynebacterium diphtheriae* [11], and Shiga toxin from *Shigella dysenteriae* and *Escherichia coli* [12]. These studies provide evidence of the value in developing bacterial toxins for potential cancer therapeutic agents.

CDT renders PCa cells sensitized to radiation

CDTs from different bacteria are capable of inducing cell-cycle arrest by activating ataxia telangiectasia mutated (ATM)-dependent DNA-damage checkpoint responses and DSBs, similar to the pathways induced by IR [13]. Given this CDT function as a radiomimetic agent, emerging and effective therapeutic modalities have been tested as treatments for different cancers [14-16]. Our previous studies also demonstrated that CDT increases the sensitivity of radio-resistant PCa cells to IR [17]. We showed that *C. jejuni* CDT enhanced radio-sensitization is attributed to the attenuation of DSB repair, long-term cell-cycle arrest in the G2/M phase. In addition, we further showed that CDT prevents the formation of autophagosomes and the inhibition of acidic vesicular organelle, which are associated with enhanced radiosensitivity in PCa cells [18]. These studies demonstrate that *C. jejuni* CDT can be developed as a potent therapeutic agent for radio-resistant cancers.

B. Preliminary results

Synthesis of biodegradable nanoparticles encapsulated with CdtB-R11

We have developed core-shell dual-imaging enabled nanoparticles that have both drug release and imaging characteristics [19]. As shown in Fig. 1, the nanoparticle core is comprised of biodegradable polyethylene glycol-gelatin (PEG-gelatin)/hyaluronan. CD44, a CSC marker, is expressed abundantly on the surface of CSC and served as a receptor for hyaluronan [20,21]. Therefore, nanoparticles containing hyaluronan should bind specificity to CSC. To prepare PEG-gelatin/hyaluronan nanoparticles that were encapsulated with CdtB-R11 (nanoparticle-CdtB), various concentrations of PEG-gelatin/hyaluronan were mixed. As shown in Table 1, mixing PEG-gelatin/hyaluronan (7.50/0.00, 7.50/1.25, 7.50/2.50, 7.50/5.00 mg/mL; 2.0 mL) were shown in the formation of complexes on the nanometer scale. The mean particle sizes of nanoparticles were ranged from 250–600 nm with negative zeta potentials, depending on the relative concentrations of PEG-gelatinand hyaluronan used. In our selected particulate system, the final concentration of PEG-gelatin/hyaluronan was 3.75/1.25 mg/mL. The particles sizes of 275.1 ± 32.6 nm possessed a significant zeta potential of -42.6 ± 5.1 mV. Therefore, this particular composition will be used to prepare the the nanoparticle-encapsulated CdtB-R11 in this proposal.

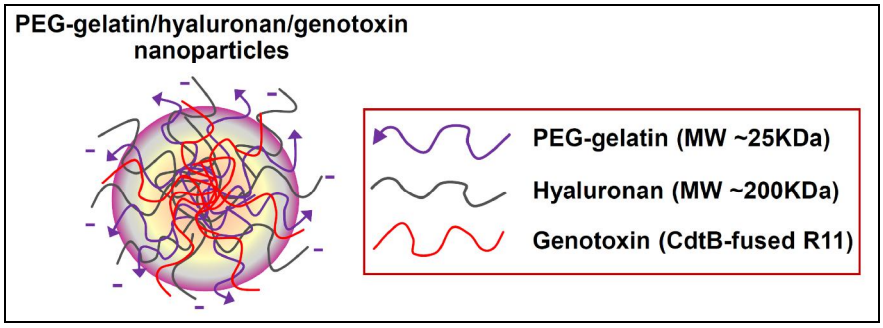

Fig. 1. Schematize the preparation and composition of PEG-gelatin/hyaluronan nanoparticles encapsulated genotoxin that are fused with R11 (CdtB, R11-CdtB, or CdtB-R11).

Table 2. Particle sizes and zeta potentials prepared with PEG-gelatin/hyaluronan nanoparticles

PEG-gelatin (mg/mL)	Hyaluronan (mg/mL)	Mean Particle Size (nm)	Zeta Potential (mV)
7.50	0.00	578.8 ± 98.6	2.5 ± 0.7
7.50	0.63	398.4 ± 25.6	-31.5 ± 3.7
7.50	1.25	275.1 ± 32.6	-42.6 ± 5.1
7.50	2.50	358.1 ± 21.6	-45.4 ± 4.9

Characterization of nanoparticle-encapsulated CdtB-fused R11 variants

As shown in Table 2, the PEG-gelatin/hyaluronan (7.50/2.505 mg/mL, 2.0 mL) mixed with CdtB, R11-CdtB or CdtB-R11 gave a mean size range of 350–390 nm, with different negative zeta potentials, depending on the different forms of recombinant CdtB that were used. This technique is promising as the nanoparticles can be prepared under deionized water at room temperature to

protect proteins from degradation in this system. In addition, the polydispersity index of nanoparticles, which were measured by dynamic light scattering, revealed a narrower distribution (polydispersity indices: 0.11 ± 0.01–0.28 ± 0.03) when compared with other formulation (Table 2 and Fig. 2). Therefore, the genotoxin and its derivatives (CdtB, R11-CdtB or CdtB-R11)-encapsulated nanoparticles prepared with this PEG-gelatin/hyaluronan will be selected in my studies.

Table 2. Particle sizes and zeta potentials of nanoparticle-encapsulated CdtB

Nanoparticles system	Mean Particle Size (nm)	Zeta Potential (mV)	Polydispersity Indices
PEG-gelatin/hyaluronan/CdtB	353.2 ± 16.3	-35.4 ± 1.2	0.28 ± 0.12
PEG-gelatin/hyaluronan/R11-CdtB	383.3 ± 19.3	-12.9 ± 1.7	0.17 ± 0.08
PEG-gelatin/hyaluronan/CdtB-R11	392.4 ± 34.7	-5.4 ± 0.7	0.21 ± 0.1

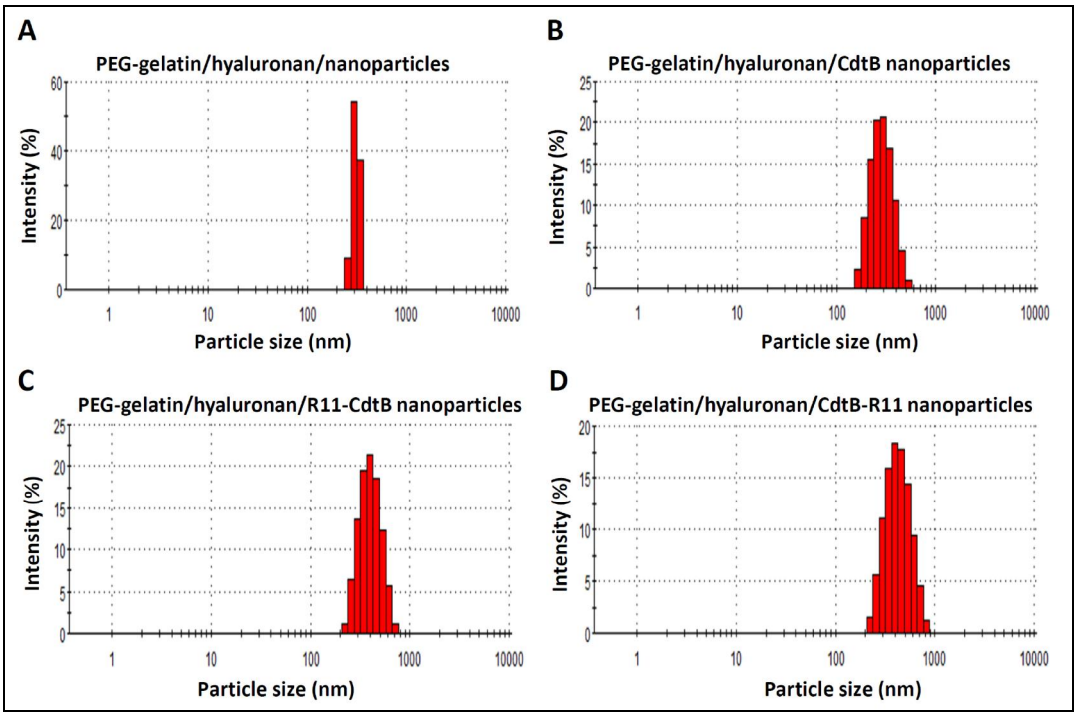

Fig. 2. Particle size distribution of prepared nanoparticles. (A) PEG-gelatin/hyaluronan; (B) PEG-gelatin/hyaluronan/CdtB; (C) PEG-gelatin/hyaluronan/R11-CdtB; and (D) PEG-gelatin/ hyaluronan/CdtB-R11.

C. Experimental designs

Radiation resistance of PCa cells, after the knockdown of DAB2IP, may be attributed to the increase in autophagy and an elevated expression of c-Myc [22]. Targeting c-Myc or autophagy to decrease their activity may impair the survival of cancer cells *in vitro* and *in vivo* [23-25]. Therefore, engineering of CdtB-R11 and encapsulate it on nanoparticles that are coated with hyaluronan, which has high affinity toward a CSC marker, CD44 [20], will be a viable approach for killing PCa cells that are resistant to radiation.

Aim 1. Evaluating biological activity of CdtB-fused with R11

CdtB contains an NLS and DNase I activity in the nucleus, causing DSB and cell death [5]. We hypothesize that CdtB-R11 proteins will target PCa cells. After entering the nucleus, CdtB-R11 causes cell cycle arrest and DNA damage. To this end, LAPC4-KD cells will be treated with CdtB-R11 derivatives (R11-CdtB, R11-sCdtB, CdtB-R11, and sCdtB-R11), R11 alone, or CdtB alone for 24 h. Cells will be fixed and stained with 20 μg/ml propidium iodide (Sigma-Aldrich) containing 1 mg/ml RNase (Sigma-Aldrich) for 1 h. The stained cells will be analyzed with a flow cytometer (BD Biosciences). The cytotoxicity of CdtB-fused R11 proteins along with CdtA/CdtB in PCa cells will be analyzed by using MTT assay, clonogenic assay, and flow cytometry. If the most effective of CdtB-R11 derivatives can be observed in these studies, this unique CdtB-R11 will be selected for the following experiments.

Aim 2. Analyzing the localization of nanoparticles-encapsulated CdtB-R11

Since CD44, a CSC marker and a receptor for hyaluronan, is highly expressed on the surface of CSC, nanoparticles containing hyaluronan should bind specifically to CSC. Herein, we will examine the functionality of the PEG-gelatin/hyaluronan nanoparticles that are encapsulated with CdtB-R11. The PCa binding, cytoplasmic distribution, and nuclear localization of the nanoparticles will be examined. To determine the localization of the nanoparticles that are encapsulated with CdtB-R11 in the cell, cells will be fixed with 4% paraformaldehyde in PBS and subjected to DAPI (1 μg/mL) counterstaining (Sigma-Aldrich). Samples will then be analyzed under a confocal microscope (Carl Zeiss). This study will demonstrate whether the nanoparticle-encapsulated CdtB-R11 enhances the specificity and promotes killing of CSC.

Aim 3. Assessing the delivery efficiency

LAPC4-KD cells will be incubated with chitosan/hyaluronan nanoparticles that are encapsulated with CdtB-R11. After 2, 6, 12, 24, and 48 h of incubation, nuclear proteins will be prepared using a nuclear extraction kit (Pierce). Protein concentrations will be determined by colorimetric assay using the Bio-Rad assay kit (Bio-Rad). The nuclear lysate (20 μg) will then be analyzed by immunoblotting. The nuclear localization of CdtB will be demonstrated by confocal microscopy. If the nanoparticles coated with CdtB-R11 can be seen in the nucleus with a high fluorescence intensity in a short time, the results will demonstrate that this engineered CdtB is delivered to CSC cells efficiently.

D. Anticipated results

The major mission of this study is to reveal the molecular basis of a bacterial genotoxin, CdtB, to sensitize radiation-induced DSB in radio-resistant PCa. We will also explore technologies for applying this bacterial genotoxin in cancer therapy. Understanding the importance of CDT-actions and molecular basis of this particular toxin will provide a useful strategy for eradicating radio-resistant PCa. We anticipate obtaining research outcomes as follows:

1. Because CDT has the function to enhance the radio-sensitivity. Therefore, engineering CdtB fused with R11 and encapsulated it on nanoparticles for the purpose of targeting at radio-resistant PCa cells can be achieved.

2. The uptake and delivery efficiency of PEG-gelatin/hyaluronan nanoparticles that are encapsulated with CdtB-R11 by PCa cells will be examined and the optimized amounts of nanoparticles will be determined. This engineered CdtB is predicted to localize on the cytoplasmic membrane and the cytosol initially after incubation and ultimately be detected in the nucleus. After reaching the nucleus, cell cycle arrest and apoptosis should occur.

3. Overcoming the radio-resistance in radio-resistant PCa through CDT and the therapeutic efficacy of genotoxin delivery nanoparticles will be validated in this proposal. Our research will impact significantly to the therapy of radio-resistant PCa.

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