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	探討細菌基因毒素增強放射線敏感性之機制			
	英文	Mechanism of bacterial genotoxin in the enhancement of radio-sensitivity			
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 108 年 1 月 1 日起至民國 108 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、整理實驗數據及撰寫研究成果與論文	80%

※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

Cytolethal distending toxin (CDT) is a genotoxin, composed of 3 subunits: CdtA, CdtB, and CdtC, produced by *Campylobacter jejuni*. It has been known that CdtA and CdtC need to interact with membrane rafts and enable the translocation of the CdtB across the cell membrane followed by delivering into the nucleus. The nuclear-translocated CdtB possesses type I deoxyribonuclease activity that can cause double-strand DNA breakage (DSB) followed by cell cycle arrest at G2/M and apoptosis. We recently demonstrated that CDT can convert radio-resistance to susceptibility in prostate cancer stem cells (PCSC). However, the potential mechanisms that may form the basis for this association are unclear. Therefore, we will investigate the mechanisms of CDT overcomes radio-resistance in PCSC. In addition, whether the autophagic pathway in response to CDT-enhanced radio-sensitivity in PCa cells will be extensively explored. The results from this study will provide novel knowledge for the application of CDT in cancer therapy.

Keywords: cytolethal distending toxin, prostate cancer, radiation therapy

A. Background and significance

Infection of *Campylobacter jejuni*

Campylobacter jejuni, a Gram-negative bacterium, which is one of the most common causative agents of food-borne infectious illnesses in humans (Butzler & Skirrow, 1979; Mead, et al., 1999). Infection by *C. jejuni* in humans usually occurs through the consumption of contaminated poultry products (Kotula & Stern, 1984; Corry & Atabay, 2001). *C. jejuni*-associated enterocolitis is typically associated with a local acute inflammatory response that involves intestinal tissue damage (Black, et al., 1988). Several bacterial virulence factors of *C. jejuni*, including adhesion molecules, flagella, and cytotoxins, have been investigated for their roles in host pathogenesis (Wooldridge, et al., 1996).

Biological function of cytolethal distending toxin (CDT)

Several bacterial virulence factors of *C. jejuni* have been studied, among those, only cytolethal distending toxin (CDT) has been characterized in detail (Lara-Tejero & Galan, 2000) and thought to be correlated with *C. jejuni*-induced local acute inflammatory responses and involved in host enterocolitis (Zheng, et al., 2008). CDT, a bacterial genotoxin, which is a tripartite protein toxin composed of 3 subunits, CdtA, CdtB, and CdtC (Lara-Tejero & Galan, 2002), encoded by an operon comprising *cdtA*, *cdtB*, and *cdtC* (Pickett, et al., 1996). CDT can be produced by several Gram-negative bacteria including *Aggregatibacter actinomycetemcomitans* (Ohara, et al., 2004), *Campylobacter jejuni* (Whitehouse, et al., 1998), *Escherichia coli* (Johnson & Lior, 1988), *Haemophilus ducreyi* (Cope, et al., 1997), and *Shigella dysenteriae* (Johnson & Lior, 1987). Previous studies showed that CdtA and CdtC are essential for mediating toxin binding to the cytoplasmic membrane of target cells (Lara-Tejero & Galan, 2001; Lee, et al., 2003; Nesic & Stebbins, 2005). Increasing evidence has demonstrated that CdtA and CdtC form a heterodimeric complex that enhances attachment of the toxin to cell membranes (Lara-Tejero & Galan, 2001; Mao & DiRienzo, 2002; Lee, et al., 2003). Upon binding to the cell membrane, CdtB is internalized into the cells and is further delivered into the nucleus (McSweeney & Dreyfus, 2004). Irrespective of the bacterial species, the nuclear-translocated CdtB contains type I deoxyribonuclease activity that can cause double-strand DNA breakage (DSB) followed by cell cycle arrest at G2/M (Nesic, et al., 2004). These insights into the biological function of the CDT holotoxin that have identified CDT as an essential factor for *C. jejuni*-induced pathogenesis in host cells (Jinadasa, et al., 2011).

Interaction of CDT and cholesterol-rich microdomains

Cholesterol-rich microdomains (known as lipid rafts) are localized on the cell membrane that contain large proportions of cholesterol, phospholipids, and glycosylphosphatidylinositol (GPI)-anchored proteins (Brown & London, 1998; Hooper, 1999). Our recent work has been demonstrated that the association of *C. jejuni* CdtA and CdtC with the host membrane is mediated in a cholesterol-dependent manner (Lin, et al., 2011). The binding of CDT subunits to the cell

membrane, nuclear delivery of CdtB, and G2/M arrest were all reduced when cellular cholesterol was depleted. Additionally, we found that the CdtC contains a cholesterol recognition/interaction amino acid consensus (CRAC) region, which is required for CdtC binding to cholesterol-rich microdomains (Lai, et al., 2013). Our results provide a molecular link between *C. jejuni* CdtC and membrane-lipid rafts through the CRAC-like region, which contributes to toxin recognition and interaction with cholesterol. We further clearly provide evidence that membrane cholesterol plays an essential role in the binding of *C. jejuni* CDT subunits to membrane rafts, which promotes the pathogenic events in host cells (Lai, et al., 2014). Apparently, these evidences reveal the interactions between *C. jejuni* CDT subunits and membrane cholesterol-rich microdomains, as well as the role of cholesterol in the CDT intoxication of host.

Prostate cancer

Prostate cancer (PCa) is the second most common cancer in men in Western countries and its incidence is rising in Taiwan (Sim & Cheng, 2005). The incidence and mortality rate of prostate cancer are stay at a relatively high level in Western countries. The recurrence of metastatic castration resistant prostate cancer (CRPC) after hormonal therapy often signifies the end stage of this disease which obtain a very high risk of bone metastases and represent a significant morbidity to patients (Eisenberger, et al., 1998). Despite the initial overwhelming responsiveness of PCa toward androgen ablation, tumor cells invariably relapse to a castration resistant (i.e., androgen-independent) state within 3-5 years that ultimately leads to mortality (Pound, et al., 1999). Although there are few options for treating patients with CRPC such as chemotherapy or radiation, patients often suffer many undesirable side effects due to their aging and deteriorated physical conditions. In order to develop safe treatment option and improve the quality of life in these patients, developing new therapeutic strategy with minimal side effects is warranted.

Rationale and significance

On the basis of the knowledge of carcinogenesis in refractory PCa, a variety of treatment approaches including chemotherapy, radiotherapy, hormonal therapy, and combinations of these modalities have been applied to patients, however, no curative regimen is available (Andreoiu & Cheng). In general, cancer cells with a high potential of distant metastasis are considered as lethal disease and accounted for the majority of mortality. Therefore, it is critical to develop a new strategy to target and eradicate the malignant phenotypes of prostate cancer cells, particular with radio-resistant phenotype.

Given the evidence that *C. jejuni* CDT can convert radio-resistance to susceptible in PCa cells, in this grant proposal, we will explore potential mechanisms that may form the basis for this association. We also will investigate the molecular mechanisms of CDT regulates autophagic pathway using cell-based and animal models. Additionally, employing small peptide as a tool for studying CDT biological function and developing into a target specific therapeutic agent in refractory prostate cancer will then be explored extensively in this study. Unveiling the molecular

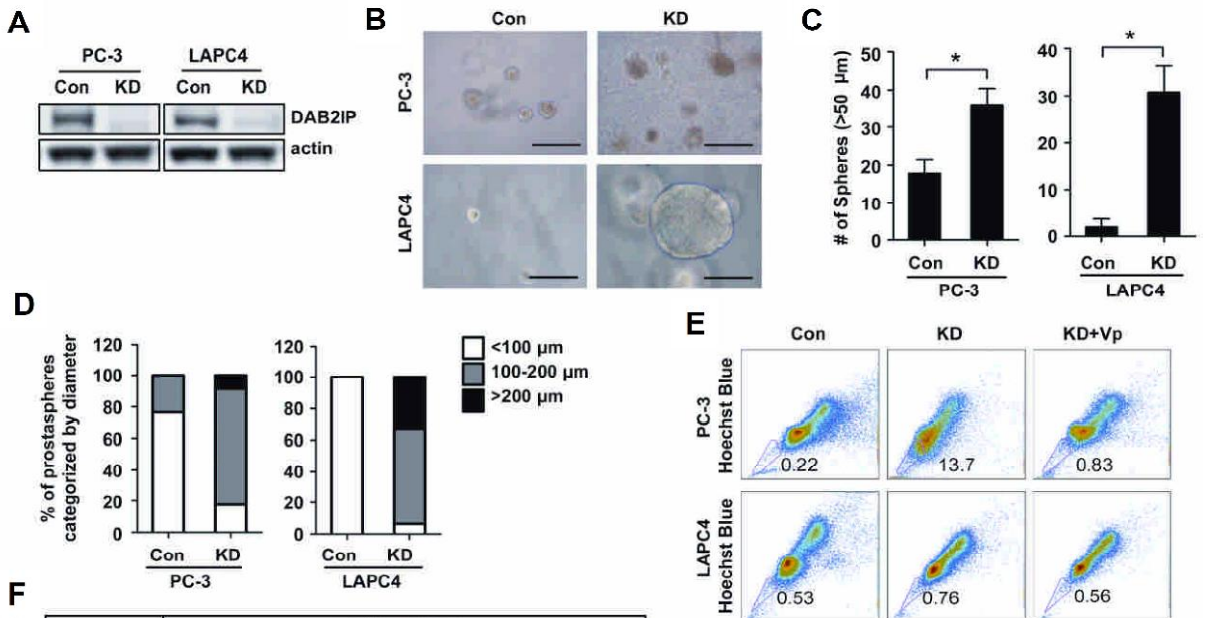
mechanism of CDT-actions, we believe this study can provide a novel therapeutic strategy for eradicating cancer stem cells of PCa.

B. Preliminary results

Our very recent study have developed chitosan/heparin nanoparticle-encapsulated CdtB, which exerts genotoxicity and enhance anti-tumor activity, represents a novel avenue for cancer therapy (Lai, et al., 2014). An additional study in our laboratory, we also reported that CDT administration significantly increased the efficacy of radiotherapy not only in cell-based model but in a xenograft mouse model (Lai, et al., 2014). Therefore, our findings indicate that this bacterial genotoxin, CDT, can be engineered to become a novel therapeutic agent, particularly, for the therapy of refractory cancers. Although the molecular mechanisms of the *Campylobacter* CDT have been explored in our previous studies (Lin, et al., 2011; Lai, et al., 2013; Lai, et al., 2014), the target specific delivery of CDT and its clinical applications have not been investigated for either *in vitro* or *in vivo* developments yet.

1. Reduces DAB2IP in PCa cells elevates the expression of stem cell markers and leads to radio-resistance

Previous studies demonstrated that a novel protein-DAB2IP is frequently lost in high-grade PCa and the function of this protein is critical for preventing PCa metastasis (Xie, et al., 2009; Xie, et al., 2010). By knocking down endogenous DAB2IP expression in CRPC cells such as LAPC4 (androgen receptor-positive [AR+]) and PC-3(AR-) (Fig. 1A), these cells were capable of forming more number and bigger size of “prostatesphere” in the suspension with matrigel compared with vector control cells (Fig. 1B). DAB2IP-knockdown (KD, shDAB2IP) cells such as PC-3-KD or LAPC4-KD formed more and bigger size of spheres than its control (Con, shVector) cells (Fig. 1C and D). In addition, Hoechst dye exclusion results indicated that KD cells increased the number of side population (SP) (Fig. 1E), indicating that KD cells express stem cell phenotypes. We then examined the tumorigenic potential of DAB2IP-KD with “stemness” potential. By inoculating different cell number combined with matrigel into ventral prostate of nude mice, as shown in Fig. 2F, clearly indicated that one single PC-3-KD cell, based on series dilution, was able to form tumor nearly 100% tumor take rate. In contrast, control cells failed to form any tumor lower than 1×10^6 cells. Similar result was also obtained from LAPC4-KD cells, indicating that the appearance of cancer stem cells (CSC) in PCa is highly related with its tumorigenicity. Accordingly, CSC is known to be resistant to radiotherapy (Kong, et al., 2010).



F

	Cells injected						
Fraction	$2X10^6$	$1X10^5$	$1X10^4$	$1X10^3$	$1X10^2$	10	1
PC-3 Con	2/8	0/8	-	-	-	-	-
PC-3 KD	8/8	8/8	8/8	8/8	8/8	6/8	15/16
LAPC4 Con	2/8	2/8	-	-	-	-	-
LAPC4 KD	8/8	8/8	6/8	4/8	4/8	2/8	2/12

*subcutaneous injection

	PC-3			LAPC4		
	$2X10^6$	10	1	$2X10^6$	$1X10^2$	10
Con	4/5	0/5	0/5	1/5	0/5	0/5
KD	5/5	5/5	3/5	4/5	5/5	4/5

*orthotopic injection

Fig. 1. Increased stemness of PCa and their tumorigenicity *in vivo*. (A-D) Characterization of stem cell phenotypes in DAB2IP KD cells. Prostate sphere formation of two PCa cells (PC-3 and LAPC4) grown in matrigel suspension condition. Scales, 20 μm. (E) Increased side population (SP) in PC-3-KD or LAPC4-KD cells. VP: verapamil. (F) Tumor incidence of Con or KD cells using orthotopic model at indicated times. Con: control; KD: knockdown. Asterisk indicated statistical significance between KD vs. Con cells ($P < 0.05$).

2. CDT increases the sensitivity of DAB2IP knockdown cells to radiation

We then assessed whether CDT enhances radiation sensitivity in the LAPC4 DAB2IP-knockdown (shDAB2IP) cells. Cells were first treated with CDT alone (0–10 nM) and the viability was determined by clonogenic assay. Our data showed that shDAB2IP cells were more susceptible to CDT than control (shVector) cells and the effect was dose-dependent (Fig. 2A). Cells were then treated with ionizing radiation (IR) alone or with a combination of CDT and IR. As shown in Fig. 2B, the shDAB2IP cells were more susceptible to combined treatment than IR alone (Fig. 2B). With increasing IR doses, CDT synergistically enhanced IR sensitivity in shDAB2IP cells but this trend was not observed in control cells.

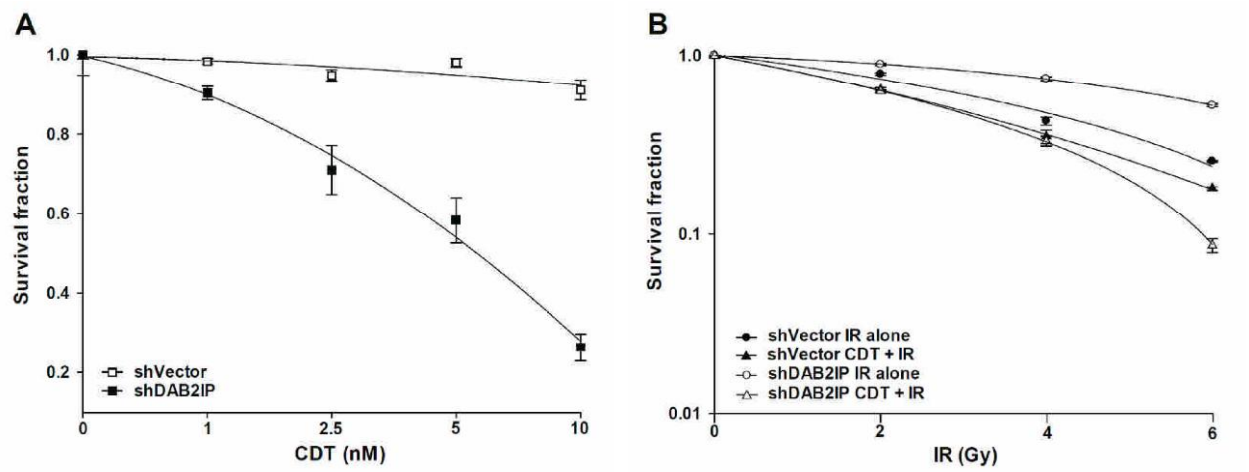


Fig. 2. CDT increases radio-sensitivity in DAB2IP knockdown PCa cells. (A) CDT, at the indicated concentration (0–10 nM), was added to shVector (Con) or shDAB2IP (KD) cells before radiation. (B) shVector or shDAB2IP cells were treated with IR (2–6 Gy) alone, or CDT (10 nM) combined with IR. After 10-days incubation, colonies were stained with crystal violet and clonogenic survival was calculated.

C. Specific aims and experimental designs

CdtB exhibits type I deoxyribonuclease activity that leads to double-strand DNA breakage (DSB) (Lara-Tejero & Galan, 2000) which may associate with increases of apoptosis or autophagy (Robert, et al., 2011). Autophagy is a lysosomal degradation pathway that eliminates damage or potentially dangerous cellular components under adverse conditions to protect organisms from metabolic stress (Kroemer, et al., 2010). Previous studies have reported that cancer cells utilized autophagy as an adaptive and context-dependent system to overcome radiotherapeutic stress (Bergmann, 2007; Apel, et al., 2008). Additionally, tumor cells in response to radiation and DNA damage, and radio-resistance may be associated with autophagy induction (Chaachouay, et al., 2011). Despite these evidences, it has been reported that autophagy may contribute to cell death after genotoxic stress (Vessoni, et al., 2013). Our recent study demonstrated that CDT, a genotoxin, can sensitize radiation-induced cell death in radio-resistant PCa cells (Lai, et al., 2014). However, whether regulation of autophagy-related pathway involved in CDT enhances radio-sensitivity in DAB2IP-deficient PCa cells remains to be investigated. Our first goal of this proposal is to explore the mechanisms of CDT overcome radio-resistance of DAB2IP-deficient PCSC.

Approach 1. Clonogenic survival assay for CDT-treated DAB2IP-deficient PCSC. To investigate the effects of CDT in radio-resistant PCa cells, C4-2 neo (DAB2IP-negative) and C4-2 D2 (DAB2IP-positive) cells will be subjected to clonogenic survival assay. Cells will be serial diluted to appropriated numbers and seeded onto 6-cm dish for 4 h. The cells will be incubated with CDT at various concentrations followed by exposed to the increasing doses of IR (0, 2, 4, 6, and 8 Gy). After 10-14 days incubation, the colonies will be fixed and stained with 0.05% crystal violet. Surviving fraction will be calculated as (mean colony counts)/[(cells inoculated) × (plating efficiency)], in which plating efficiency is defined as (mean colony counts)/(cells inoculated for unirradiated controls) as described previously (Kong, et al., 2010).

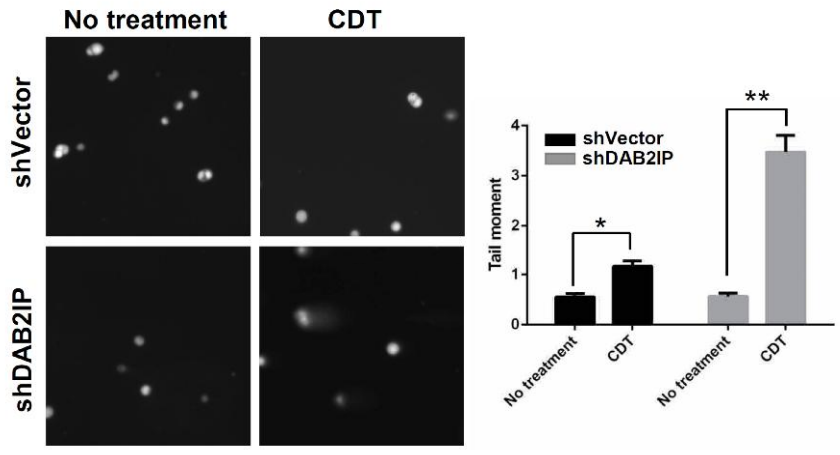


Fig. 3. CDT-induced DNA damage in DAB2IP-deficient PCa cells. LAPC4 cells transfected with either shVector or shDAB2IP were either left untreated or treated with CDT (200 nM) for 24 h. The cells were then electrophoresed and stained. The labeled DNA was visualized under a fluorescence microscope. The tail moment was quantified for each cell; these data are presented as histograms in the right-hand panels. **, $P < 0.01$.

Approach 2. Evaluation of DNA damage by comet assay. Our preliminary data showed that the comets were significantly increased in DAB2IP-knockdown cell lines following treatment with CDT relative to the comets in untreated cells (Fig. 3). In contrast, CDT only slightly increased the comets in the DAB2IP-positive (shVector) cells. To test our finding that CDT enhanced IR-induced cytotoxicity in DAB2IP-deficient PCa cells was mediated by DSB, the comet assay will be performed. Cells (C4-2, LAPC4, and PC3) with or without DAB2IP will untreat or treat with 200 nM CDT follow by irradiation at room temperature in ambient air using a 137Cs source (Mark 1-68 irradiator, JL Shepherd & Associated) at a dose rate of 2 Gy/min. The cells will be collected and subjected to Comet Assay (Trevigen). The nuclei will stain with propidium iodide (Sigma-Aldrich) and the comets will observe by using a fluorescence microscope (Carl Zeiss). The tail moments (tail DNA% × tail length) will be quantified from 50 randomly selected cells.

Approach 3. Western blot analysis of effector molecules that involved in apoptotic pathway. To ascertain CDT-induced radio-resistant PCa cell death is through apoptosis mechanism including triggering the mitochondrial apoptotic pathway or ER-stress, we will measure the change in expression of Bcl-2 family proteins (including Bcl-2, Bax and Bak), p53, calpains, caspase-9 and caspase-3. Proteins of the cellular lysates will be resolved on SDS-PAGE and transferred to Immobilon polyvinyldifluoride membranes (Millipore). The blots will be blocked and then probed with antibodies against Bcl-2, Bax, caspase 3/9, PARP, and calpains (Cell Signaling) for 1 h at room temperature. The blots will be subsequently incubated with anti-rabbit peroxidase conjugated secondary antibody (Santa Cruz) for 1 h at room temperature and will be visualized by enhanced chemiluminescence using X-OMAT LS film (Eastman Kodak).

Possible problems and alternatives. We don't expect any technical problem. To strengthen our observation, we will examine the effect of CDT on the radio-sensitivity of C4-2 neo (DAB2IP-negative) and C4-2 D2 (DAB2IP-positive) cells. In addition to clonogenic survival assay, we will determine apoptosis using flow cytometry for analysis of sub-G1 population, PI/AnnexinV assay (Calbiochem), or TUNEL assay (Roche Diagnostics); the stained cells will then be analyzed using a FACScan and the Cellquest program (Becton Dickinson).

Anticipated outcomes. We expect that the radiation-sensitizing effect of CDT can be observed in DAB2IP-negative cells. Moreover, increasing cell death in radio-resistant PCa by CDT through the enhancement of IR-induced DSB and induction of apoptotic pathway will also be explored in this aim.

D. Expected results and goals

The major mission of this study is to unveil the molecular basis of a bacterial genotoxin–CdtB to sensitize radiation-induced DNA double-strand break (DSB) in PCSC. We will innovate novel technologies to apply the bacterial genotoxin in the cancer therapy. Understanding the importance of CDT-actions and molecular basis of this toxin, we are confident to provide a novel strategy for eradicating PCSC. Along with basic research of this study, we also expect to publish these findings in high-impact journals as well as transfer our outcomes to start-up biotech industries.

(1) We will address scientific issues related to the molecular mechanisms of CDT to sensitize radio-resistant in refractory PCa and innovation of delivery systems for PCSC-targeting therapy.

(2) A multi-disciplinary research will be established including molecular biology, cancer biology, and nanotechnology, which will comprehensive support to the biology community.

(3) Most importantly, we will train graduate students and research assistants to be trained as independent and skillful biologists who will be the future human resource for the biomedical research and biotech industry in Taiwan.

(4) We expect to have one or two high-quality publications, which contains state-of-art technologies, tools, and new information of biomedical research.

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