Hyaluronic Acid Nanoparticles-encapsulated Bacterial Genotoxin for Targeted Therapy of Radioresistant Prostate Cancer

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Research

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Abstract

**Background:** Prostate cancer (PCa) is one of the most commonly diagnosed cancers in men and usually becomes refractory because of recurrence and metastasis. CD44, a transmembrane glycoprotein, serves as a receptor for hyaluronic acid (HA) and has been found to be abundantly expressed in cancer stem cells (CSCs) that often exhibit a radioresistant phenotype. Cytolethal distending toxin subunit B (CdtB), produced by *Campylobacter jejuni*, is a genotoxin acts as a type I deoxyribonuclease (DNase I), which is responsible for creating DNA double-strand breaks (DSBs). Nanoparticles loaded with antitumor drugs and specific ligands that recognize cancerous cell receptors are promising methods to overcome the therapeutic challenges.

**Results:** In this study, PCa cells with DOC-2/DAB2 interactive protein (DAB2IP) knockdown were employed to imitate the CSC with radioresistant phenotype. We further prepared HA-decorated nanoparticles-encapsulated CdtB (HA-CdtB-NPs) and investigated the targeted therapeutic activity in radioresistant PCa cells. Our results showed that HA-CdtB-NPs sensitized radioresistant PCa cells by enhancing DSB and causing G2/M cell-cycle arrest, without affecting the normal prostate epithelial cells. These results demonstrate that HA-CdtB-NPs possess maximum target-specificity and delivery efficiency of CdtB into the nucleus, and enhance the effect of radiation in radioresistant PCa cells.

**Conclusions:** These findings indicate that HA-loaded CdtB nanoparticles exert target-specificity accompanied with radiomimetic activity, which can be developed as an effective agent for overcoming radioresistance in PCa.

Background

Prostate cancer (PCa) is a threatening malignancy that demonstrates an escalating trend in both the incidence and mortality rate worldwide in the recent years [1]. Patients may develop several non-specific symptoms such as urinary retention, urinary hesitancy, nocturia, and hematuria [2]. Because of the high heterogeneity of PCa, the relationship between certain symptoms and cancer development is rather weak and undefined [3]. However, attributed to the advancement in diagnostic tests, more people opt for clinical management during the early phases. Treatment strategies for PCa include surgery, chemotherapy, hormone therapy, and radiation therapy [4]. Among these, radiation therapy is considered as an effective remedy for localized cancers, which improves prognosis when combined with other appropriate treatment modalities [5, 6]. Therefore, the unpredictable emergence of radioresistance may result in a setback for successful treatments. Even worse, development of radioresistance often leads to tumor metastasis or relapse [7]. To overcome this problem, a radiation sensitizer is likely to be the solution.

Cytolethal distending toxin (CDT), a genotoxin produced by *Campylobacter jejuni* (*C. jejuni*), causes DNA double-strand breaks (DSBs) [8]. CDT comprise three subunits: CdtA, CdtB, and CdtC, which is encoded by the consecutive genes *cdtA*, *cdtB*, and *cdtC*, respectively [9]. The working subunit, CdtB, acts as a DNase I, which is responsible for creating DSB, cell cycle arrest at the G2/M phase [10], thereby inducing cell
distension and ultimately cell death [11]. However, CdtB cannot traverse the cell membrane alone and therefore, its entry into the cytoplasm requires the binding of CdtA and CdtC to the cell membrane. With the help of the nuclear localization signal (NLS), CdtB is subsequently translocated into the nucleus [12]. Given the similarity of CDT with radiation, wherein both elicit cell death by causing DSBs, it is highly possible for CDT to be an ideal radiation sensitizer.

Although CDT appears to be a promising cure for radioresistant PCa, the specific delivery of CDT to PCa cells remains a critical issue. This challenging task can be overcome by utilizing the CD44-overexpressing nature of PCa cells [13]. CD44 is a transmembrane glycoprotein which mediates extracellular adhesion and signal transduction [14]. In addition, CD44 has been involved in tumor progression and metastasis, drug resistance, and radiation resistance of cancer cells [15, 16]. Several studies have demonstrated that using hyaluronic acid (HA)-decorated nanoparticles to encapsulate various therapeutic drugs could effectively target CD44-overexpressing cells and subsequently deliver the encapsulated agents into those cells [17-21]. HA, also known as hyaluronan, is a naturally occurring non-sulfated glycosaminoglycan, with characteristics such as biocompatibility, non-immunogenicity, and biodegradability, making it an ideal material for targeting CD44+ cancer cells [21].

In this study, HA-decorated nanoparticles-encapsulated CdtB (HA-CdtB-NPs) were developed and extensively studied for their characteristics, including the particle size, loading efficiency, and zeta-potential. PCa cells with DOC-2/DAB2 interactive protein (DAB2IP) knockdown were chosen to imitate the cancer stem cells (CSC) with radioresistant phenotype [22]. After treating PC3 DAB2IP-knockdown (PC3-KD) cells with HA-CdtB-NPs, the drug efficacy, cytotoxicity, and delivery efficiency were assessed.

**Materials And Methods**

**Antibodies and reagents**

Antibodies against cleaved-PARP was purchased from Proteintech (Chicago, IL). Antibodies specific to phospho-H2AX and phospho-CHK2 were purchased from Cell Signaling (Danvers, MA). Antibodies against β-actin, proliferating cell nuclear antigen (PCNA), and rabbit horseradish peroxidase (HRP)-conjugated antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against CD44 was purchased from Novus Biologicals (Centennial, CO). Other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Purification of CDT subunits**

Recombinant His-tagged CdtA, CdtB and CdtC subunits were constructed as described in our previous study [23]. A single colony of *Escherichia coli* BL21-DE3 containing *cdtA, cdtB, cdtC* expression plasmids, respectively, were picked and cultured in LB broth with ampicillin (100 μg/mL) at 37°C. *E. coli* harboring *cdtA, cdtB, cdtC* recombinant plasmids were then induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 5 h for which containing *cdtA and cdtB*, and at 16°C for 16 h for which containing *cdtC,*
respectively. Recombinant proteins were purified by metal affinity chromatography (Clontech, CA, USA) and analyzed by SDS-PAGE and western blot assay.

**Preparation of HA-decorated nanoparticle-encapsulated CdtB**

Recombinant CdtB was purified according to the protocol as described above. To prepare HA-decorated nanoparticle-encapsulated CdtB, a simple ionic gelation with magnetic was stirred at room temperature. The determination of the optimal preparation conditions was performed by examining different proportions of HA/arginine-chitosan (HA/Arg-CS). In brief, aqueous HA (4.00 mg/mL, 0.50 mL) was added into aqueous Arg-CS with various concentrations (1.00, 2.00, 3.00 or 4.00 mg/mL, 0.50 mL), and then the solutions were gently shaken for 30 min at 37°C. After collecting the prepared HA/Arg-CS NPs produced by centrifugation, a Zetasizer instrument was used to measure the particle size, polydispersity index, and zeta potentials. Meanwhile, the NP suspension was placed onto a mesh copper grid and positively stained with osmium tetroxide to observe their morphology under transmission electron microscopy.

Next, different concentrations of CdtB were encapsulated in nanoparticles to determine the optimal condition for preparing CdtB-loaded HA/Arg-CS NPs. CdtB dissolved in deionized water (3.00, 2.00, 1.00 mg/mL; 0.25 mL) was mixed with HA solution (8.00 mg/mL; 0.25 mL) to form CdtB/HA aqueous mixed solutions (1.50:4.00, 1.00:4.00, and 0.50:4.00 mg/mL, 0.50 mL). The CdtB/HA solutions (0.50 mL) were added to the optimal concentration of Arg-CS in deionized water (0.50 mL) and stirred at 37°C for 30 min. After centrifugation, the amount of free CdtB in the supernatant was detected by a protein concentration assay (Bio-Rad, CA, USA) and protein-loading efficiency of the NPs calculated from the following equation:

\[
\text{Loading efficiency} = \frac{\text{total CdtB protein} - \text{free CdtB protein}}{\text{total CdtB protein}} \times 100\%
\]

**Cell culture**

Human prostate epithelial cell line (PZ-HPV-7) was derived from the peripheral zone of a benign prostate and cultured as described previously [24]. The methods that used to knockdown endogenous DOC-2/DAB2 interactive protein (DAB2IP) in PCa cells were described previously [25]. PC3 knockdown DAB2IP (PC3-KD) cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT) with 5% fetal bovine serum (FBS) (Hyclone) containing puromycin (0.4 μg/mL), 1× penicillin/streptomycin, and G418 (800 μg/mL) in the environment of 37°C and 5% CO₂.

**Analysis of cell viability**

PC3-KD cells were seeded in 96-well plates and treated with 0 nM, 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, and 1,000 nM of HA/Arg-CS (HA-NPs), CDT holotoxin and HA-CdtB-NPs, respectively. After incubation
for 48 h, cells were treated with 100 μL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution in 37°C for 2 h. Cell viability were analyzed by measuring the ability of viable cells reducing MTT (Sigma-Aldrich) to formazan [26].

**HA-CD44 localization and DNA double strand break (DSB) by immunofluorescence**

PC3-KD cells were seeded onto cover glasses and incubated for 24 h. For DSB analysis, cells were first treated with 2 Gy ionizing radiation (IR), then exposed to 200 nM of HA-NPs, CDT holotoxin and HA-CdtB-NPs, respectively. Cells were subsequently fixed with 1% paraformaldehyde for 1 h, permeabilized by 0.1% triton X-100 for 15 min. For HA-CD44 colocalization assay, cells were then treated with primary antibody against CdtB. For DSB analysis, cells were treated with anti-pH2AX and anti-53BP1 at the dilution of 1:200, followed by treatment with secondary antibody at the dilution of 1:500 and 4',6-diamidino-2-phenylindole DAPI (0.2 μg/mL). The stained cells were observed using Laser Scanning Confocal Microscope (LSM780, ZEISS, Germany).

**Analysis of CdtB in the nuclear extraction**

PC3-KD cells were treated with 100 nM HA-NPs, CDT holotoxin, and HA-CdtB-NPs, followed by incubation for 0, 0.5, 1, 3, and 6 h. The nuclear proteins were isolated using a nuclear extraction kit (Pierce, Rockford, IL), as described previously [23]. CdtB level in the nuclear fraction was then analyzed by western blot assay.

**Western blot analysis**

PC3-KD cells were seeded onto 60 mm dish and incubated in 37°C for 24 h, then treated with medium (mock), IR, HA-NPs accompanied by IR, CDT holotoxin accompanied by IR, and HA-CdtB-NPs accompanied by IR, respectively, for 24 h and 48 h. Cell lysates were centrifuged at 12,000 rpm for 20 min at 4°C. After quantification, samples were resolved by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore, MA). The PVDF membrane was blocked and probed with primary antibodies against (ADP-Ribose) P polymerase (PARP), pH2AX, pATM, pCHK2, and β-actin in the condition of gentle shaking. After an overnight incubation at 4°C, the membrane was then probed with horseradish peroxidase-conjugated secondary antibody (Millipore) in room temperature for 1 h. The proteins of interests were detected using ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) and were visualized using an Azure c400 system and AzureSpot Analysis Software (Azure Biosystems, USA) by following the manufacturer’s instructions.

**Cell cycle analysis by flow cytometry**

PC3-KD cells were incubated with ICRF-193 (2 μg/mL), HA-CdtB-NPs (50 nM, 100 nM, and 200 nM), and HA-NPs alone for 24 h. The cells were washed with PBS, centrifuged for 1,000 rpm in room temperature for 5 min. Subsequently, the prepared cells were resuspended in hypotonic buffer (0.2 mg/mL RNase A, 20μg/mL propidium iodide, and 0.1% Triton X-100). Analysis of cell cycle were performed by flow cytometry (Becton Dickinson, CA).
Comet assay

Experiment protocols followed instructions by Trevigen CometAssay® Kit (Trevigen, Gaithersburg, MD, USA). PC3-KD cells were seeded in 60 mm dish and respectively treated with medium (mock), HA-NPs, CDT holotoxin, and HA-CdtB-NPs with three of the latter accompanied with IR. After incubation for 24 h, PC3-KD cells \(1 \times 10^5/mL\) were first mixed with LMAgarose with the proportion of 1:10 and placed onto CometSlide™ with complete coverage of sample area, then incubated the slide at 4°C in the dark for 30 min. Cells were then immersed with 4°C lysis solution at 4°C for 30-60 min and followed by alkaline unwinding solution at 4°C in the dark for 1 h. CometSlide™ were placed in alkaline electrophoresis solution and set power supply to 21 volts then applied voltage for 30 min and washed by distillation-distillation \(\text{H}_2\text{O (ddH}_2\text{O)}\) and 70% alcohol. After CometSlide™ were dried and stained by CYBR® Gold, cells were observed using Leica CTR 4000 (Leica, Germany).

Statistical analysis

The analysis among multiple groups was carried out by means of one-way analysis of variance. The post-hoc test was used to measure the statistical significance of differences between two groups. Statistical analyses and chart drafting were carried out using the GraphPad Prism 6 software (GraphPad Software Inc, CA, USA).

Results

Preparation and characterization of CDT and HA-CdtB-NPs

Purified recombinant CDT subunits and synthesized HA-CdtB-NPs were analyzed by SDS-PAGE (Fig. 1A and 1B). The NPs were constructed with negatively charged HA and positively charged arginine-chitosan (Arg-CS). As shown in Table 1, HA:Arg-CS used in distinct concentrations (2.0:0.5, 2.0:1.0, and 2.0:2.0 by mg/mL) formed complexes on the nanometer scale, with the exception of the concentration 2.0:1.5 mg/mL. The diameters of the prepared NPs were in the range of 220-300 nm (Table 1 and Fig. 1C), and the negatively or positively charged values were dependent on the relative concentrations of HA and Arg-CS. When the amount of the positively charged Arg-CS sufficiently exceeded that of the negatively charged HA (HA:Arg-CS = 2.0:2.0 mg/mL), the NPs had Arg-CS exposed on their surfaces and thus, had a positive surface charge. In contrast, when the amount of the negatively charged HA significantly exceeded that of the positively charged Arg-CS (HA:Arg-CS = 0.5:2.0 or 1.0:2.0 by mg/mL), some of the excessive HA molecules were entangled onto the surfaces of the obtained NPs. Thus, the resulting NPs displayed a negative surface charge. The HA/Arg-CS concentration of 2.0:0.5 mg/mL produced the mean particle size of 225.64 ± 14.65 nm with a maximum negative zeta potential of −38.79 ± 4.51 mV \((n = 5)\). Therefore, the NPs prepared with this specific composition were used for the following studies.

In order to prepare the CdtB-encapsulated NPs, various concentrations of CdtB were mixed with HA/Arg-CS encapsulated mixture solution. As shown in Table 2, the CdtB:HA:Arg-CS in distinct concentrations
showed a mean size range of 250-300 nm, with different negative potential values. When the concentration of CdtB:HA:Arg-CS is 0.75:2.00:0.50 mg/mL, the loading efficiency of CdtB in NP is 48.97 ± 5.12%, its particle size is 273.78 ± 17.37 nm, zeta potential value is -39.89 ± 9.65 mV. In addition, the polydispersity index of NPs measured using dynamic light scattering (DLS) revealed a narrow distribution (polydispersity index: 0.27 ± 0.06) and the morphology of the prepared NPs along with CdtB-encapsulated NPs remained spherical and smooth-shaped on transmission electron microscopy observation (Fig. 1D). Therefore, the HA-decorated NPs encapsulating CdtB with a negative surface charge at a CdtB:HA:Arg-CS concentration of 0.75:2.00:0.50 mg/mL were used for the remaining studies.

**HA-CdtB-NPs induce radioresistant PCa cell death**

Loss of DOC-2/DAB2 interactive protein (DAB2IP) expression in PCa cells leads to the development of radioresistance [25]. We therefore utilized DAB2IP-knockdown PCa cells (PC3-KD) as the research platform to investigate the therapeutic effects of HA-CdtB-NPs on radioresistant PCas. We first analyzed the CD44 expression in PCa cells and normal prostate epithelial cells. As shown in Fig. S1, higher CD44 expression was observed in PC3-KD cells than in PZ-HPV-7 cells. For evaluating the ability of HA-CdtB-NPs to induce PCa cell death, we performed an MTT assay to analyze the effect of HA-NPs, CDT holotoxin, and HA-CdtB-NPs on the cell viability of the PC3-KD cells and PZ-HPV-7 cells. To determine the most ideal condition for the following experiments, we treated PC3-KD cells with various concentrations of the HA-CdtB-NPs and found that the viable cell numbers dropped to approximately half at a concentration of 100 nM (Fig. 2A). Meanwhile, CDT holotoxin treatment also exhibited a reduction effect in a concentration-dependent manner; however, it was less effective than HA-CdtB-NPs. Furthermore, it was denoted that HA-NPs barely affected both PC3-KD and PZ-HPV-7 cells (Fig. 2B). Most importantly, PZ-HPV-7 cells did not show any evident response to HA-CdtB-NPs treatment, which indicated that HA-CdtB-NPs are able to specifically target PCa cells.

**Delivery of HA-CdtB-NPs in PCa cells**

To assess the delivery efficiency of HA-CdtB-NPs into PCa cells, we treated PC3-KD cells with HA-CdtB-NP for different time periods and observed the colocalization of CD44 and HA. As shown in Fig. 3A, CD44-HA colocalization could be noted at 0.5, 1, and 3 h, with the peak level of colocalization observed at 1 h. In addition, we performed a western blot analysis for checking the nuclear translocation of CdtB and the expression levels were also quantified (Fig. 3B, C). The highest expression of CdtB in the nucleus was observed at 6 h after HA-CdtB-NPs treatment. Interestingly, PC3-KD cells treated with HA-CdtB-NPs demonstrated significantly higher expression of CdtB in the nucleus than those treated with CDT holotoxin. The results showed that HA-CdtB-NPs attached to PC3-KD cells primarily at 1 h after the treatment, and at 6 h, most CdtB entered the nucleus. Our results indicated that HA-CdtB-NPs manifested better delivery efficiency of CdtB into the nucleus than CDT holotoxin, which corresponded with the cell viability assay.

**HA-CdtB-NPs enhance IR-induced DSB**
To examine whether HA-CdtB-NPs regain the sensitivity of PC3-KD cells toward IR, three different procedures that allow the visualization of DSBs were performed, and four conditions were included: mock treatment, HA-NPs/IR cotreatment, CDT holotoxin/IR cotreatment, and HA-CdtB-NPs/IR cotreatment. First, in the comet assay, comet-like structures were observed in the cells for all treatment conditions except for the mock treatment (Fig. 4A). Nevertheless, the HA-CdtB-NPs/IR cotreatment displayed a brighter and longer tail than the other treatments. Second, expression levels of γ-H2AX, p-CHK2, and cleaved-PARP were analyzed using western blot (Fig. 4B). HA-NPs/IR cotreatment manifested almost similar low expression as the mock treatment. Although the γ-H2AX and p-CHK2 expressions in CDT holotoxin/IR and HA-CdtB-NPs/IR treatments are similar, expression levels of γ-H2AX, p-CHK2, and cleaved-PARP are still the highest in HA-CdtB-NPs/IR treatment among all the treating conditions. Finally, as shown by the immunofluorescence staining, the extent of DSB is indicated using the number of foci of γ-H2AX and 53BP1 (Fig. 5). Similar to the previous results, the foci were observable in all treatment conditions except for mock-control, and HA-CdtB-NPs/IR cotreatment solely had maximum foci formation. These results reveal that HA-CdtB-NPs were the most effective and could synergistically enhance IR-induced DSB in radioresistant PCa cells.

**HA-CdtB-NPs induce cell cycle arrest**

Since DSB elicits the spontaneous activation of DNA damage response, which is likely to arrest the cell cycle, we further characterized the effect of HA-CdtB-NPs on the cell cycle distribution. For the control group and HA-NPs treatment group, merely 6.29% and 6.25% of cells were arrested at the G2/M phase (Fig. 6A, B). ICRF-193 treatment served as a positive control, which arrested 89.82% of cells at the G2/M phase. An apparent increase in the population in the G2/M phase was detected with HA-CdtB-NPs treatment; moreover, the increase in HA-CdtB-NPs concentration also increased the population at the G2/M phase. These results demonstrate that HA-CdtB-NPs possessed a better activity that induced DSBs and cell cycle arrest at the G2/M phase.

**Discussion**

Radiotherapy has long been a powerful strategy against cancer cell proliferation; however the malignant cancer cells often find ways to bypass cell death [7, 27-29]. The utility of combinatorial treatment modalities, specifically radiosensitizers in cancer therapy, have been investigated in the early 1970s [30]. Recently, nanotechnology has pioneered a bright road towards the development of innovative cancer therapy platform [31, 32]. Owing to the possibility of modification of nanoparticle composition and surface coating, the functions and specificity of nanoparticles could be more flexibly customized [33-35].

In our previous study, we have developed nanoparticles of HA and polyethylene glycol-gelatin (PEG-gelatin) to specifically target CD44⁺ PCa cells [36]. It has been revealed that CD44 expressing cells exhibit stem cell-like properties [37, 38], and could be an ideal target for selectively killing CSC [39]. Mounting evidence has suggested the likelihood of CSC involvement in radioresistance [40-42], supporting the utility of CSC-targeted radiosensitizer to improve the efficacy of radiotherapy.
Many bacterial toxins have been extensively studied for their promising clinical applications [43-47]. A previous study has shown that human gingival squamous carcinoma cell line Ca9-22 transfected with *Actinobacillus actinomycetemcomitans*–CdtB expressing plasmid, manifested G2 phase arrest and cell growth inhibition [48]. Additionally, in our previous work, chitosan/heparin nanoparticle-encapsulating CdtB was proved to be effective in causing G2/M phase cell cycle arrest in gastric cancer cells and finally induced cell death [49]. The potential of CDT as a radiosensitizer also has been tested in PCa models [50]. As described before, CDT exerts a similar effect as radiation via CdtB entry into the nucleus, but with the precondition of CdtA and CdtC binding to the cell surface. To minimize the size of the particles for better cellular uptake efficiency and tumor permeability, nanoparticles allowing intracellular delivery were designed, and only CdtB (without CdtA and CdtC) was purified and encapsulated into the nanoparticles. In addition, PEG-gelatin nanoparticles possess the preferable advantages such as biocompatibility, biodegradability and cost-effectiveness [51, 52]. Gelatin is a denatured protein obtained from animal structural collagen [53], which can properly form a complete structure with CdtB; in addition, the PEG-modification provides the protection against enzymatic damage and mononuclear phagocytosis system [54], which allows a sustained circulation.

In this study, we attempted to analyze the potential of CDT as a radiosensitizer, and further exploited progressive nanotechnology to develop a novel combinatorial treatment for PCa. To determine the binding activity of HA-CdtB-NPs, we visualized the localization of HA-CdtB-NPs using immunofluorescence and found that most HA-CdtB-NPs attached to the target cells as early as 1 h after treatment. Moreover, the amount of CdtB entry into the cytosol and subsequently the cell nuclei with the HA/PEG-gelatin delivery system was the highest at 6 h after treatment. Notably, the results also indicated that HA-CdtB-NPs have a better CdtB delivery efficiency than CDT holotoxin. Hence, CdtB has the potential to cause cancer cell death by directing DSB, which makes it an effective radiosensitizer.

The enhancement of radiosensitivity was further examined by the combined effect of HA-CdtB-NPs and IR. DSB formation and the phosphorylation of downstream proteins such as CHK2 and γ-H2AX confirmed the activation of ATM-dependent DNA damage signaling pathway [55]. PCa cells treated with IR showed positive results, while HA-CdtB-NPs/IR presented the most synergistic effect. Furthermore, after 24 h treatment with HA-CdtB-NPs, a large proportion of PCa cells were paused at the G2/M phase, which has been revealed to be the most vulnerable stage for radiation [56-58].

Although our results show that HA-CdtB-NPs possess the potent therapeutic effect in radioresistant PCa cells, further investigations are required for a more realistic medical application. For instance, even though previous research showed that HA has an equivalent cancer cell cytotoxicity as that of anti-CD44 antibodies, CD44 does not serve as the only receptor for HA and it may not be a specific biomarker for PCa cells [39]. Therefore, *in vivo* studies aimed at the analysis of biodistribution, circulation time, antitumor activity and side effects of the HA-CdtB-NPs should be performed.

**Conclusion**
In this study, we developed an HA-based nanoparticle delivery system to carry CdtB into PCa cells. Our results demonstrated that HA-CdtB-NPs possess activity similar to CDT holotoxin, but with superior advantages. The potential effects include maximum target-specificity and delivery efficiency of CdtB into the nucleus, and enhancement of the effect of IR in radioresistant PCa cells. Our results provide a novel strategy to employ HA-nanoparticles encapsulated with CdtB as a radiation sensitizer, which can be developed as a potent therapeutic agent for radioresistant PCa.

**Declarations**

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**Competing interests**

The authors declare that they have no competing interests.

**Author's contributions**

Conception or design of this work: HL, JTH, CHC, YHL, and CHL; Experimental study: CYC, YRL, HYW, YJL, and YFC; Data analysis and interpretation: CLH, CJL, and UGL; Writing the manuscript: CYC, YRL, CHC, YHL, and CHL; Final approval: all authors. †CYC and YRL were equally contributed to this work.

**Consent for publication**

Not applicable.

**Availability of supporting data**

All the data and materials presented in the current study along with additional files are available.

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Tables

Table 1. Characteristics of prepared NPs constructed with differential HA:Arg-CS concentrations.
Table 2. Characteristics of HA-NPs encapsulating varying CdtB concentrations.

| CdtB:HA:Arg-CS (mg/mL)† | Mean Particle Size (nm) | Polydispersity indices | Zeta Potential Value (mV) | CdtB Loading Efficiency (%) |
|--------------------------|-------------------------|------------------------|---------------------------|-----------------------------|
| 0.75:2.00:0.50           | 273.78 ± 17.37          | 0.27 ± 0.06            | -38.89 ± 9.65             | 48.97 ± 5.12                |
| 0.50:2.00:0.50           | 261.43 ± 18.95          | 0.28 ± 0.08            | -35.48 ± 7.32             | 45.79 ± 6.51                |
| 0.25:2.00:0.50           | 248.62 ± 20.16          | 0.21 ± 0.09            | -35.13 ± 4.83             | 43.87 ± 4.98                |

†Different encapsulated CdtB concentrations resulted in distinct particle sizes, polydispersity indexes, zeta potentials, and CdtB-loading efficiencies of HA-NPs (n = 5).

Figures
Figure 1

Characterization of HA-CdtB-NPs. SDS-PAGE analysis of (A) each recombinant CDT subunit and (B) HA-CdtB-NPs. Transmission electron micrographs of (C) HA-NPs and (D) HA-CdtB-NPs. Scale, 200 nm.
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Figure 2

HA-CdtB-NPs inhibit PCa cell proliferation. (A) PC3-KD cells were treated with HA-NPs, CDT holotoxin or HA-CdtB-NPs at the indicated concentrations (0-1000 nM) for 48 h. (B) PZ-HPV-7 and PC3-KD cells were treated with HA-NPs, CDT holotoxin or HA-CdtB-NPs at the concentration of 100 nM for 48 h. Cell viability was assessed using MTT assay. Statistical significance was evaluated using one-way ANOVA with post hoc test (*, p < 0.05).
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Delivery of HA-CdtB-NPs into PCa cells. (A) PC3-KD cells were treated with HA-CdtB-NPs-GFP for 0, 0.5, 1, 3, and 6 h. The colocalization of CD44 and HA on the cell membrane was observed. Scale, 10 μm. (B) PC3-KD cells were exposed to 100 nM HA-NPs, CDT holotoxin, and HA-CdtB-NPs, then incubated for the indicated times. The nuclear fraction from cell lysates was prepared and CdtB in the nucleus was analyzed by western blotting. Proliferating cell nuclear antigen (PCNA) was used as a loading control for nuclear protein. (C) The expression of CdtB in the nucleus was quantified for each time point. Statistical significance was evaluated using one-way ANOVA with post hoc test (*, p < 0.05).
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HA-CdtB-NPs induce cell cycle arrest and apoptosis. (A) PC3-KD cells were mock-treated, exposed to ICRF-193 or HA-CdtB-NPs (50, 100 and 200 nM) and incubated for 48 h. Cell cycle distribution based on DNA content was analyzed using flow cytometry. (B) The percentage of cells at G2/M phase were calculated. Statistical significance was evaluated using one-way ANOVA with post hoc test (*, p < 0.05).
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