ISSN: 0975-3583, 0976-2833 VOL4, ISSUE 3, 2013

Doxorubicin-Loaded Aptamer S3-Linked DNA Tetrahedrons for Targeted Therapy of Nasopharyngeal Carcinom.

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Abstract

Objective: Our research group previously identified CD109 as the target of aptamer S3 in nasopharyngeal carcinoma (NPC). This study aimed to develop a targeted delivery system by conjugating aptamer S3 to DNA tetrahedrons (DTs) loaded with doxorubicin (Dox), and to evaluate the potential of S3-DT-Dox for targeted NPC therapy.

Methods: Aptamer S3-conjugated DTs were synthesized and loaded with Dox. The effects of S3-DT-Dox on NPC cells were investigated using laser confocal microscopy, flow cytometry, and MTS assays. A nude mouse tumor model, established using NPC 5-8F cells, was used to examine the in vivo anti-tumor activity of S3-DT-Dox through fluorescent probe labeling and hematoxylin-eosin staining.

Results: The synthesized S3-DTs exhibited high purity and stability. S3-DT specifically recognized 5-8F cells and NPC tissues in vitro. A 1:20 ratio of S3-DT to Dox resulted in optimal Dox loading efficiency. The maximum drug release rate (0.402±0.029) was achieved 48 hours after S3-DT-Dox was incubated in PBS. S3-DT did not alter Dox toxicity in 5-8F cells but reduced Dox toxicity in non-target cells. In vivo, S3-DT-Dox specifically targeted transplanted tumors in nude mice and inhibited tumor growth, with minimal damage to normal tissues.

Conclusion: This study demonstrates the potential and safety of S3-DT-Dox for targeted delivery to NPC cells and inhibition of NPC development.

Keywords: Nasopharyngeal carcinoma, Aptamer S3, CD109, 5-8F cells, DNA tetrahedrons, Doxorubicin.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy originating from the uppermost part of the pharynx, covering the surface of the soft palate and the base of the skull [1]. According to the GLOBOCAN 2020 database, which provides epidemiological data for 36 cancers across 185 countries, NPC accounts for over 133,000 new cases and approximately 80,000 deaths annually [2]. This malignancy exhibits significant ethnic and geographic distribution, with particularly high prevalence in southern China, Southeast Asia, northern Africa, and the Middle East [3]. Epstein-Barr virus (EBV) infection is widely recognized as a potential etiological factor for NPC [4]. In addition to EBV, genetic susceptibility and chemical carcinogens also contribute to NPC pathogenesis [5]. Recent decades have seen significant advances in understanding the genomic, epigenomic, and immune landscapes of NPC, providing a theoretical foundation for developing new biomarkers and therapeutic targets [6]. Concurrent chemoradiotherapy remains the standard treatment for locoregionally advanced NPC, but high-dose radiation or chemotherapy can lead to inevitable acute and late toxicities

ISSN: 0975-3583, 0976-2833 VOL4, ISSUE 3, 2013

[7]. Doxorubicin (Dox) is a commonly prescribed and effective antitumor agent. The combination of ifosfamide and doxorubicin is effective in treating recurrent and metastatic NPC [8]. However, doxorubicin faces major challenges, including cardiotoxicity and drug resistance [9]. Therefore, new approaches to enhance doxorubicin efficacy while mitigating toxicity and resistance are urgently needed. Modifications of antitumor drug delivery systems have garnered significant attention as they can facilitate the specific delivery of chemotherapeutic agents to tumor cells, thereby minimizing dose-related and off-target toxicities [10]. For example, folate-conjugated polyethylene glycol (PEG)-phosphatidylethanolamine (PE)-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles [1] have shown promise as a doxorubicin delivery system capable of enhancing antitumor efficacy [11]. Aptamers, nucleic acid-based macromolecules with high affinity and specificity for their targets, hold great potential as targeting moieties for nanocarriers. They can specifically recognize cancer cells and deliver antitumor drugs, potentially increasing therapeutic efficiency through multivalent effects [12, 13]. Recent studies have demonstrated that chimeric aptamers can deliver doxorubicin to cancer cells, improving therapeutic efficacy and reducing off-target cytotoxicity [14]. DNA tetrahedrons (DTs), nanostructures formed from four short single-stranded DNA molecules, can serve as carriers for multiple antitumor agents, such as floxuridine, camptothecin, and platinum drugs, delivering them to tumor cells when appropriately designed [15]. Several studies have shown that doxorubicin can be intercalated between DNA base pairs and effectively delivered to drug-resistant breast cancer cells using DNA tetrahedrons, with or without aptamer modification [16, 17]. Aptamer-guided DNA tetrahedrons loaded with doxorubicin increased cytotoxicity to PTK7-positive cells while having a minor effect on normal cells, demonstrating potential application in T-cell acute lymphoblastic leukemia [18]. However, few studies have explored this delivery system in NPC. Our research group previously identified CD109 as the target of aptamer S3 and a potential NPC biomarker [19]. In this study, we developed a delivery system in which doxorubicin was loaded into CD109-recognizing aptamer S3-conjugated DNA tetrahedrons (S3-DT-Dox) and evaluated its effect on NPC.

Materials and Methods

Ethics Statement: Animal experiments were approved by the Animal Care Committee MMCHRI and conducted in compliance with relevant ethical guidelines and regulations. Experiments involving clinical samples were approved by the Ethics Committee of MMCHRI. Informed consent was obtained from all participants providing clinical samples.

Cell Culture: The human nasopharyngeal carcinoma (NPC) cell line 5-8F was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The human normal nasopharyngeal epithelial cell line NP69 (ATCC, Manassas, Virginia, USA) was cultured in keratinocyte serum-free medium (KSFM) at 37 °C with 5% CO2.

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Aptamer: Aptamer S3, targeting CD109 on 5-8F cells, was used. This aptamer was previously selected from synthetic DNA libraries by cell-based systematic evolution of ligands by exponential enrichment (CELL-SELEX) in our laboratory and verified for its specificity.

Human Sample Collection: NPC tissue samples were obtained from the tissue bank at MMCHRI. Patients undergoing biopsy at the Otolaryngology Department of MMCHRI Hospital provided the samples. Nasopharyngitis tissues were collected from patients undergoing biopsy for nasopharyngitis.

Synthesis of DT, S3-DT, DT-Cy5, S3-DT-Cy5, DT-Dox, and S3-DT-Dox:

The base sequences of the four single-stranded DNA molecules used to form the DNA tetrahedrons (DTs).

- **DT Synthesis:** 10 μL of each single-stranded DNA (A, B, C, and D) at 10 μM were annealed in 10×TAE buffer (10 μL) and ultrapure water (50 μL) at 95 °C for 10 minutes, followed by natural cooling. The final DT concentration was 1 μM.
- **S3-DT Synthesis:** 10 μL of each single-stranded DNA (A, B, C, and D) and S3 fragments (all at 10 μM) were annealed in 10×TAE buffer (10 μL) and ultrapure water (40 μL) at 95 °C for 10 minutes, followed by natural cooling. The final S3-DT concentration was 1 μM.
- **DT-Cy5 Synthesis:** 10 μL of each single-stranded DNA (A, B, and C) and D-Cy5 fragments (all at 10 μM) were annealed in 10×TAE buffer (10 μL) and ultrapure water (50 μL) at 95 °C for 10 minutes, followed by natural cooling. The final DT-Cy5 concentration was 1 μM.
- **S3-DT-Cy5 Synthesis:** 10 μL of each single-stranded DNA (A, B, and C), D-Cy5, and S3 fragments (all at 10 μM) were annealed in 10×TAE buffer (10 μL) and ultrapure water (40 μL) at 95 °C for 10 minutes, followed by natural cooling. The final S3-DT-Cy5 concentration was 1 μM.
- **DT-Dox Synthesis:** 50 μL of each single-stranded DNA (A, B, C, and D) at 400 μM were annealed in 10×TAE buffer (40 μL) and ultrapure water (160 μL) at 95 °C for 10 minutes, followed by natural cooling. The resulting DT solution (50 μM) was incubated with 4 μL of 100 mM doxorubicin (Dox) at 37 °C for 3 hours. The final solution contained DT (50 μM) and Dox (1 mM).
- **S3-DT-Dox Synthesis:** 50 μL of each single-stranded DNA (A, B, C, and D) and S3 fragments (all at 400 μM) were annealed in 1×TAE buffer (40 μL) and ultrapure water (110 μL) at 95 °C for 10 minutes, followed by natural cooling. The resulting S3-DT solution (50 μM) was incubated with 4 μL of 100 mM Dox at 37 °C for 3 hours. The final solution contained S3-DT (50 μM) and Dox (1 mM).

Agarose Gel Electrophoresis:

A 2.5% agarose gel was used to analyze the synthesized DNA constructs. 50 bp DNA ladder was used as a marker. Samples of A chain, A+B chain, A+B+C chain, DT, and S3-DT were mixed with loading buffer and loaded onto the gel. Electrophoresis was performed at 120 V and 400 mA for 30 minutes.

Particle-Size Analysis:

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The particle size of S3-DT and DT (1 pmol in 1 mL double-distilled water) was measured using a Malvern particle size analyzer.

Stability Detection of DT and S3-DT:

- DT and S3-DT (1 μM) were incubated in McCoy's 5A complete medium at 37 °C. Samples were collected at 0, 2, 4, 6, 8, 10, 12, and 24 hours and analyzed by gel electrophoresis.
- DT and S3-DT (1 μM) were incubated with FBS (1:1) at 37 °C. Samples were collected at 1, 3, 5, and 7 hours and analyzed by gel electrophoresis.

Laser Confocal Analysis:

5-8F and NP69 cells (1 × 10^4) were incubated with Cy5-labeled S3, S3-DT, or DT (250 nM) at 37 °C for 3 hours. Cells were washed and imaged using a Leica laser confocal microscope (63× lens, excitation 633 nm, emission 650-750 nm).

Flow Cytometry:

- For cellular recognition, 5-8F and NP69 cells (4 × 10^5) were incubated with Cy5-labeled S3, S3-DT, or DT (250 nM) at 37 °C for 3 hours. Cy5 fluorescence was measured by flow cytometry.
- For cellular uptake, 5-8F and NP69 cells were incubated with Dox, DT-Dox, or S3-DT-Dox (2 μM Dox) at 37 °C for 1.5 hours. Cells were washed, fixed, and analyzed by flow cytometry.

Fluorescence Detection of NPC Tissues:

Paraffin sections of NPC and nasopharyngitis tissues were dewaxed, hydrated, and subjected to antigen retrieval. Sections were blocked and incubated with biotin-modified S3, S3-DT, or DT (1 μM) at 37 °C for 2 hours. Streptoavidin-modified ZnCdSe/ZnS quantum dots were added, and fluorescence was detected using a fluorescence microscope (excitation 488 nm, emission 600 nm).

Optimum Loading Ratio of Dox in DT:

Dox fluorescence quenching was used to determine loading efficiency. 2 μM Dox was incubated with varying concentrations of S3-DT or DT (0-100 nM) at 37 °C for 3 hours. Dox fluorescence was measured using a fluorometer (excitation 480 nm, emission 540-700 nm).

Release Efficiency of Dox:

S3-DT-Dox complexes (100 nM S3-DT, 2 μM Dox) were prepared and incubated in PBS. Released Dox was collected at various time points using ultrafiltration, and fluorescence was measured.

MTS Cell Viability Assay:

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5-8F and NP69 cells (5 × 10^3) were incubated with Dox, DT-Dox, or S3-DT-Dox (0.01-40 μM Dox) for 2 hours, followed by 48 hours in culture medium. Cell viability was measured using the MTS assay.

Western Blot:

Protein samples were prepared, separated by SDS-PAGE, and transferred to membranes. Membranes were incubated with antibodies against CD109 and GAPDH, and protein expression was detected by chemiluminescence.

Results

CD109 Expression in 5-8F and NP69 Cells: Aptamer S3 specifically recognizes and binds to the CD109 antigen on the surface of 5-8F cells but not NP69 cells. Western blot analysis confirmed that CD109 protein expression was detectable in 5-8F cells but not in NP69 cells (Fig. 1).

Synthesis and Stability Analysis of DT and S3-DT: DNA tetrahedrons (DTs) were synthesized from four single-stranded DNA molecules through complementary base pairing (Fig. 2A). Agarose gel electrophoresis confirmed the successful synthesis of DTs and S3-DTs, showing the expected molecular size order: S3-DT > DT > A+B+C > A+B > A (Fig. 2B). Particle size analysis using a Malvern particle sizer revealed that DTs had a particle size of 14.23 ± 3.79 nm (PDI 0.293 ± 0.05) and S3-DTs had a particle size of 23.02 ± 6.54 nm (PDI 0.378 ± 0.06), both with a single peak, indicating high purity and homogeneity (Fig. 2C). Stability tests, involving incubation in complete medium and 1:1 FBS, showed that DTs and S3-DTs were stable for up to 12 hours in complete medium and 5 hours in 1:1 FBS, with slight degradation observed at longer incubation times (Fig. 2D, E). DT stability was slightly reduced with S3 ligation, but both DT and S3-DT exhibited good overall stability.

Identification of NPC Cells and Tissues by S3-DT: Cy5-labeled DTs (DT-Cy5), S3 (S3-Cy5), and S3-DTs (S3-DT-Cy5) were used to assess cellular recognition. Laser confocal microscopy showed that DT-Cy5 exhibited red fluorescence in both 5-8F and NP69 cells, while S3-Cy5 and S3-DT-Cy5 showed red fluorescence only in 5-8F cells, indicating specific recognition of 5-8F cells by aptamer S3 and S3-DT (Fig. 3A). Flow cytometry results corroborated these findings, demonstrating that S3-Cy5 and S3-DT-Cy5 exhibited significantly higher fluorescence intensity in 5-8F cells compared to NP69 cells, while DT-Cy5 showed no significant difference (Fig. 3B). Fluorescence detection of clinical tissues revealed that S3 and S3-DT targeted NPC tissues but not nasopharyngitis tissues, as evidenced by red fluorescence in NPC tissues only (Fig. 3C).

Effects of Dox-Loaded S3-DT on NPC Cells: Dox fluorescence quenching assays indicated that Dox loading on S3-DT and DT increased with increasing S3-DT or DT concentrations, reaching an optimal loading ratio of 1:20 (S3-DT/DT:Dox). Dox release assays showed a

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gradual increase in Dox release over time, reaching a maximum release rate of 0.402 ± 0.029 at 48 hours. Flow cytometry analysis demonstrated that S3-DT-Dox specifically delivered Dox into 5-8F cells but not NP69 cells, while free Dox and DT-Dox delivered Dox into both cell lines (Fig. 4C). MTS assays showed that S3-DT-Dox exhibited higher cytotoxicity to 5-8F cells compared to NP69 cells, while free Dox and DT-Dox showed no significant difference (Fig. 4D, E). The IC50 value of S3-DT-Dox in 5-8F cells was significantly lower than that of free Dox and DT-Dox, whereas in NP69 cells, it was significantly higher.

Effects of S3-DT-Dox on Growth of Transplanted Tumors and Tissue Damage in Nude Mice: In vivo imaging showed that S3 and S3-DT, but not DT, targeted transplanted tumors in nude mice, with S3-DT exhibiting higher tumor accumulation. Tumor growth inhibition assays revealed that S3-DT-Dox significantly inhibited tumor growth compared to free Dox and DT-Dox, while free Dox and DT-Dox significantly inhibited tumor growth compared to DPBS. Histological analysis of heart, liver, spleen, kidney, and lung tissues showed that S3-DT-Dox caused less damage to normal tissues, especially the kidney, compared to free Dox and DT-Dox.

Discussion:

Advances in management, radiotherapy, chemotherapy, and tumor staging have improved the overall prognosis for nasopharyngeal carcinoma (NPC) [20]. Among chemotherapeutic agents, doxorubicin (Dox) has shown efficacy in treating recurrent metastatic NPC [21]. In this study, we developed a targeted delivery system for Dox, demonstrating that S3-DT-Dox can specifically recognize NPC cells and deliver Dox, maintaining its cytotoxic effect on tumor cells while minimizing damage to normal cells. In vivo experiments further confirmed that S3-DT-Dox targeted tumor grafts, exhibited superior tumor-inhibitory activity compared to free Dox or DT-Dox, and reduced Dox toxicity to normal tissues.

Aptamers, such as the E3 aptamer, are considered ideal for drug targeting due to their high affinity and small size [22]. Recent work has shown that a modified aptamer-bound microfluidic chip can achieve a ~90% capture rate of NPC cells [23]. Our in vitro and in vivo findings support the excellent ability of S3-DT-Dox to recognize NPC cells and inhibit tumor growth, while exhibiting reduced toxicity to normal cells and organs.

Various systems have been developed to enhance Dox efficacy and reduce its toxicity. For instance, PLGA-PEG-folate nanoparticles encapsulating Dox, perfluorooctyl bromide (PFOB), and indocyanine green (ICG) have been proposed as a promising targeted chemotherapeutic carrier, traceable by 19F MRI or NIR imaging [24]. Similarly, GE11-engineered graphene quantum dots (GQDs@GE11)/cisplatin (CDDP)/Dox nanoprobes have demonstrated specific tumor targeting and inhibition of tumor cell proliferation [25]. Furthermore, a 2-methoxyestradiol-emulsified drug delivery system has shown promise in enhancing Dox uptake by adriamycin-resistant breast cancer MCF-7 cells [26].

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DNA tetrahedrons (DTs) have also emerged as promising nanocarriers for tumor-targeting drugs. L-DNA tetrahedrons have been shown to target cancer cells, enhance Dox accumulation in tumors, and reduce Dox-induced cardiotoxicity [27]. DTs have demonstrated potential as efficient carriers for Dox delivery to drug-resistant breast cancer cells [16]. MUC1-targeting DT-conjugated AS1411 aptamer nanocarriers of Dox have also shown promise in reducing drug cytotoxicity and resistance in breast cancer [17]. Consistent with these findings, our study demonstrated that DT-Dox could deliver Dox to 5-8F cells. However, only S3-DT-Dox specifically recognized 5-8F cells, effectively delivering Dox without significantly damaging normal cells and tissues.

This study demonstrates that S3-DT specifically recognizes NPC cells, enhances Dox delivery, maintains Dox toxicity on NPC cells, and reduces Dox toxicity to normal cells and organs. This modified delivery system holds therapeutic promise for improving the prognosis of NPC patients.

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