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Chemotherapeutic, Toxin, and Therapeutic Protein Delivery via Nucleolin Aptamer-functionalized Nanoplatforms for Targeted Cancer Therapy

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Abstract

Nucleolin is a protein abundantly present in the nucleolus, but its expression on the surface of cells is potentially associated with various types of malignancies. So far, several nucleolin-targeting strategies, including the nucleolin-targeting peptide, anti-nucleolin pseudopeptides, anti-nucleolin antibodies, and the anti-nucleolin aptamer, AS1411, have been developed and investigated in different types of studies. The AS1411 aptamer has been known as the outstanding approach for targeting nucleolin with superior specificity and therapeutic potential in comparison with other targeting strategies. In this review, we highlight different nucleolin-targeting strategies for the targeted delivery of chemotherapeutic drugs, proteins with therapeutic potential, and toxins.

Keywords: Nucleolin, AS1411, Aptamer, Cancer, Nanoparticles, Drug Delivery

1. Context

The cell surface expression of nucleolin is correlated with the metabolic and proliferative activity and tumorigenic potential of cancer stem cells (CSCs) and various cancer cell lines (1). As known, CSCs are highly tumorigenic and have essential roles in tumor relapse (2). This fact supports the idea of considering nucleolin as a therapeutic target (2). Both cell surface and cytoplasmic forms of nucleolin have roles in cancer progression, as proven by their high level of expression in cancer cells compared to non-transformed cells (3). This fact makes nucleolin a promising and easily accessible target for cancer therapy (3). Moreover, nucleolin overexpression at the cell surface is only restricted to tumor cells, and major organs such as the liver, heart, spleen, and lungs lack the cell surface expression of nucleolin (3). Therefore, the potential of nucleolin as both a prognostic marker and a therapeutic target is an undeniable fact.

Aptamers are DNA- or RNA-based oligonucleotides that harbor unique and steady three-dimensional structures in various environments, including inside cells. They are typically selected from a synthetic chemical combinatorial library of 10^{14} - 10^{15} different oligonucleotides designed for binding to the desired target molecules through the approach of systematic evolution of ligands by exponential enrichment (SELEX) and its variations, including immunoprecipitation-coupled SELEX, capture-SELEX, cell-SELEX, capillary electrophoresis-SELEX, atomic force microscopy-SELEX, and artificially expanded genetic information system-SELEX (4).

The AS1411 aptamer, which has a sequence of 5′-GGTGGTGGTGGTTGTGGTGGTGGTGG-3′, is a high-affinity nucleolin-binding DNA aptamer that forms a G-quadruplex structure and is resistant to enzymatic degradation of the reaction of serum enzymes (5). Even though the exact action mechanism of AS1411 is still undiscovered, early experimental evidence has demonstrated that the anticancer activity of AS1411 is attributed to its quadruplex forming ability towards nucleolin which shows high affinity (6). Besides, AS1411 binds to the nucleolin, abundantly present at the surface of cancer cells, and then actively enters the cells through micropinocytosis, followed by its transportation to the cell nucleus (7). The overexpression of nucleolin in cancer cells allows for the incremental uptake of AS1411, leading to its consequent

high intracellular concentration and elevated tumor cytotoxicity. This elevated level of cytotoxicity against tumor cells can be efficiently used as a platform for targeted drug delivery (8). The binding of AS1411 to nucleolin prevents the induced expression of proliferative and anti-apoptotic genes (9). In detail, AS1411 is capable of inhibiting NF-kB (10) and BCL-2 signaling (11) and inducing cell cycle arrest (12) and tumor suppressor gene expression (13).

Just like in the case of the conjugation of chemotherapeutic agents with aptamers, siRNA and miRNA can be covalently conjugated with aptamers to form aptamer-siRNA or aptamer-miRNA chimeras. Studies have demonstrated that the downregulation or upregulation of genes of interest is feasible in aptamer-mediated gene therapy (14, 15). It has been shown that these chimeras are capable of enhancing the efficacy and specificity of several cancer treatment drugs (16). Therefore, they can provide a novel combinational strategy for cancer therapy (16). Moreover, in aptamer-mediated target cell biotherapy, aptamers could grant agonistic or antagonistic effects upon their interaction with many cell surface receptors and biomarkers. This interaction can lead to a disruption in specific biologic functions of target cancer cells that can result in their programmed cell death. In a nutshell, aptamers are a new member of the targeted cancer therapy family with a great market value and strong applicability in many biomedical fields, from in vivo tumor imaging and in vitro cancer cell detection to targeted cancer therapy, which could replace the old methods or work alongside them. In this review, we highlight the capability and applicability of the nucleolin aptamer for the development of targeted cancer therapeutics based on chemotherapeutics, toxins, and therapeutic proteins.

2. Targeted Delivery Systems Functionalized with AS1411

2.1. Delivery of Chemotherapeutic Drugs

Despite considerable advances in nanotechnology-based cancer treatments, their wide clinical application is still limited due to several challenges, including their poor physiochemical properties such as complicated preparation processes, low drug loading capacity, and poor stability, as well as their severe side effects in the liver, spleen, and lungs (17). The severe side effects observed in the mentioned organs are mainly due to the nonspecific uptake of nanoparticles by mononuclear phagocytic cells present in these organs (17). These side effects could also be attributed to the insufficient intracellular accumulation of anticancer agents and, consequently, poor pharmacological activities in tumor cells caused by the endocytosis process difficulties of these nanoparticles (17). Redirecting nanoparticles using aptamers with significant specificity towards a particular cancer-associated target such as nucleolin can be an efficient method to overcome some of the mentioned hurdles.

As proposed by Zhang et al., the incorporation of Apt-TPGS copolymer into micelle surfaces improves cancer cell recognition through the presence of nucleolin on the plasma membrane of cancer cells, while the encapsulation of PTX in the Apt-mixed micelles results in the quick release of the drug in a weakly acidic environment with a pH of 5.5 (18). Besides, PTX/Apt-mixed micelles exhibit significantly increased internalization only into cancer cells rather than healthy ones, which is because of their Apt-nucleolin interaction-mediated enhanced transmembrane ability leading to a significantly increased tumor accumulation of PTX and consequently, elevated cytotoxicity, G2/M phase arrest, and tumor growth inhibition. In general, this dual-functional Apt-mixed micellar system can serve as a promising and potent targeted drug-delivery system for the treatment of various cancer types (18).

Also, a study developed AS1411-functionalized poly (L-γ-glutamyl-glutamine)-paclitaxel (AS1411-PGG-PTX) nanoconjugates for specific delivery of PTX to nucleolin overexpressing cancer cells, which could result in the combination of the active targeting and optimized solubilization of paclitaxel (19). This platform demonstrated to be a promising targeting delivery strategy for glioblastoma treatment (19). These nanoconjugates induced apoptosis in most tumor cells and exhibited a pronounced anti-glioblastoma effect with prolonged median survival time, which mediated the binding and endocytosis of AS1411-PGG-PTX nanoconjugates to glioblastoma and neovascular endothelial cells, due to the nucleolin overexpression of these cells (19) .

Additionally, other researchers have reported the development of a negatively charged surface-modified drug delivery system to overcome the issue of inefficient cell uptake of such systems, which limits their therapeutic performance (20). They reported the fabrication of receptor-mediated surface charge inversion nanoparticles, composed of undecylenic acid-modified thermally hydrocarbonized porous silicon (UnTHCPSi) nanoparticle cores, which are sequentially modified with polyethylenimine (PEI) and methotrexate (MTX) and finally functionalized with AS1411 (UnTHCPSi-PEI-MTX@AS1411) for the aim of enhancing cellular uptake of these nanoparticles by nucleolin overexpressing cells (20). The interaction between AS1411 and the cell-surface nucleolin causes the disintegration of the surface negative charge, leading to the subsequent surface charge inversion and MTX exposure (20). This results in the enhancement of the cellular uptake of these nanoparticles (20). Furthermore, this nanosys-

tem shows efficient performance for combination therapy with a considerable inhibition ratio while loaded with sorafenib (SFN) (20).

The cytotoxic effects and high antitumor activity of Doxorubicin (DOX) on tumor cells are considerably limited by the slow-releasing process of the drug, which is due to the lack of a definitive triggering mechanism and the development of multidrug resistance (MDR) that is mediated by an increase in the drug efflux due to the expression of plasma membrane P-glycoprotein (P-gp) transporters (21). One of the few strategies aiming at solving this problem is the use of drug delivery vehicles, which can be taken up by cancer cells through receptor-mediated endocytosis (22). One of these delivery vehicles is liposomes that are small spherical-shaped artificial vesicles with at least one lipid bilayer. Liposomes, created from cholesterol and naturally non-toxic phospholipids, are considered promising systems for drug delivery due to their size, hydrophobicity, and hydrophilicity, as well as their biocompatibility (23). They can be equipped with the AS1411 aptamer, which makes them potential drug carriers towards various cancer cells.

Here, we describe the development of an efficient thermoresponsive liposomal drug delivery system capable of rapid drug release triggering (24). Particular types of DOX-resistant cancer cells that overexpress nucleolin receptors are targeted by anti-nucleolin aptamer-functionalized liposomes (AS1411 liposomes) that contain DOX and ammonium bicarbonate (ABC; NH_4HCO_3). The highly efficient encapsulation of DOX through remote-loading is mediated by the transmembrane gradient, which itself is generated by the encapsulation of a bubble-generating agent, ammonium bicarbonate, into the liposomal system (22). The functionalization of the liposomes with AS1411 promotes their affinity and specific binding to cell-surface nucleolin while improving their uptake rate by tumor cells in comparison with plain liposomes, which encounter difficulties entering the cells. Upon the introduction of mild hyperthermia (approximately 42°C), which can be locally produced using ultrasound energy, microwave, radiofrequency, or magnetic hyperthermia, the decomposition of the ABC, which is encapsulated in the aqueous compartment of the liposomes, is started (22). Once the ABC is decomposed, permeable defects are created in the lipid bilayers of the liposomes due to the immediate generation of CO_2 bubbles, which will eventually facilitate the rapid intracellular release of DOX, yielding a cell-killing threshold-exceeding concentration (22).

In comparison with free DOX or passively targeted plain liposomes, the targeting of nucleolin-expressing tumor cells with thermoresponsive AS1411-functionalized liposomes can significantly increase the accumulation of DOX in the tumor interstitium, thus mediating the inhibition of tumor growth and minimizing systemic side effects such as cardiotoxicity. Considering the important role of intratumoral drug release in combating cancer, these functionalized liposomes can serve as potentially effective and targeted delivery vehicles for cancer therapy since they exhibit the capabilities of minimizing the MDR effect of tumor cells through bypassing the P-gp-mediated drug efflux (22).

Nucleolin-specific AS1411-functionalized hydrogels can also be used as drug carrier platforms for the controlled encapsulation and release of various anticancer medications such as DOX in targeted cancer therapy with an excellent cancer cell recognition ability (25). Hydrogels are three-dimensional networks of physically or chemically cross-linked individual hydrophilic polymer chains capable of holding a large amount of water while sustaining their particular structure (26). Hydrogels have been one of the most attractive polymeric materials for the development of controllable and sustained drug-release systems (26). This particular application of hydrogels is due to their pharmaceutically ideal characteristics, such as biocompatibility and biodegradability profiles, and non-toxicity (26). Furthermore, the application of smart hydrogels is mainly because of their susceptibility to physiochemical changes in response to external stimuli such as temperature, light, pH fluctuations, and molecular recognition (26).

Here, we describe an AS1411-functionalized hydrogel developed by Wang et al. (25). The synthesis process of this AS1411-functionalized hydrogel starts with two pieces of acrydite-modified oligonucleotides termed acrydite-modified oligonucleotides A (S-A) and acrydite-modified oligonucleotides B (S-B), which are copolymerized in the presence of acrylamide resulting in the conjugation of linear DNA-polyacrylamide conjugates that are called PS-A and PS-B, respectively. In a mixture of equal proportions of S-A and S-B, the grafted polymers exhibit transparent liquid form. Furthermore, the sequences of S-A and S-B are complementary to an adjacent site of the AS1411 aptamer, allowing for the cross-linking of the linear polyacrylamide polymers through the hybridization of S-A and S-B with the aptamer sequence in the presence of the aptamer. It is notable to state that doxorubicin is encapsulated within the gel network during the formation of the hydrogel. Furthermore, increasing the polymer solution viscosity is a reflection of the incremental pattern of the cross-linking ratio, which reaches higher levels as the hybridization process goes on. The releasing process of the encapsulated doxorubicin in the hydrogels will be mediated through the competitive binding of the AS1411 aptamer to the target molecule of nucleolin, leading to a reduction in the density of the cross-linking and the consequent dissolu-

tion of the gel. This nucleolin-induced gel dissolution, which causes the loaded drug to release, maximizes the therapeutic efficacy of this aptamer-functionalized hydrogel, which is designed as a controlled drug release system while concurrently minimizing various adverse side effects. Alongside controlled drug release, the biomedical applications of this multifactorial platform can also be extended into different areas, including gene delivery and biomedical diagnosis (25). Table 1 comprehensively lists AS1411-functionalized chemotherapeutic drug delivery nanoplatforms developed for targeted cancer therapy.

2.2. Delivery of Toxins with Therapeutic Benefit

Toxins are poisonous substances that can be of biological origins such as living organisms or microorganisms or of chemical origins such as synthetic toxins (45). To date, toxins have been applied in various biomedical fields, such as cancer treatment (45). Targeting specific molecules such as nucleolin that exhibit over-expression profiles in cancer cells using a toxin-delivery system offers a highly cytotoxic strategy for the selective elimination of cancer cells while sparing normal and healthy tissue cells. Selective and redirected toxin delivery to nucleolin can be achieved through different targeting mechanisms, such as using a synthetic nucleolin antagonist or nucleolin-specific aptamer, AS1411 (46). Here, we describe an elaborate strategy for targeted cytolysis of nucleolin-expressing cells using a nucleolin-specific aptamer-mediated toxin delivery system.

Melittin is the principal component of honeybee venom, which has been extensively investigated because of its particular characteristics enabling it to be utilized as an anti-bacterial and anticancer agent (47). The potential cytotoxicity and growth inhibitory effects of this cationic linear peptide have been demonstrated on a wide spectrum of tumor cells such as in the lungs, liver, renal, breast, cervical, prostate (47). These characteristics of melittin are mainly attributed to its protein aggregation causing properties, its ability to induce changes in cell membrane potential, and its capability of causing severe deformation in the structure of membrane phospholipid bilayers and intracellular organelles, consequently leading to irreversible cellular damage and cell death (48). The phospholipid packing disruption ability of melittin is majorly accredited to its potential role in the activation of phospholipase A2 and its interactions with cell membrane phospholipid bilayer due to the hydrophobicity of its amino-terminal and hydrophilicity of its carboxyl-terminal region, which subsequently leads to the formation of lethal membrane pores through lateral movements (48). Altogether, the abovementioned lytic and apoptotic properties make this potent venom peptide a suitable candidate for targeted therapy of a range of human cancer types while coupled with a safe and effective delivery carrier. The covalent conjugation of melittin to the anti-nucleolin aptamer, AS1411, using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide) (EDC/NHS) allows for selective and precise targeted delivery of aptamer-melittin complex to the cell surface of nucleolin over-expressing cancer cells through a receptor-ligand mediated interaction. This fabricated toxin-delivery platform of aptamer-melittin conjugate significantly reduces the cytotoxic effects of free melittin towards normal cells with no nucleolin receptor overexpression profile and increases the cytotoxicity of melittin towards nucleolin positive cancerous cells (47).

2.3. Delivery of Proteins with Therapeutic Values

Researchers have designed and developed an autonomous AS1411-functionalized DNA nanorobot with the blood coagulation protease thrombin within its inner cavity to be specifically transported and presented to tumor cells (49). While intravenously injected, these DNA nanorobots specifically bind to nucleolin on the surface of tumor-associated endothelial cells, and since the aptamer acts as both a targeting domain and a molecular trigger for the mechanical opening of the DNA nanorobot, the release of the encapsulated thrombin is mediated (49). In turn, thrombin activates coagulation at the tumor site, which leads to the induction of localized intravascular thrombosis, resulting in tumor infarction and cell necrosis (49).

This novel DNA nanorobot is constructed from single-stranded M13 phage genomic DNA linked together by staple strands to structurally form a rectangular DNA sheet on the surface of poly-T oligonucleotide-conjugated thrombin molecules which are loaded by hybridization with poly-A sequences that protrude from the DNA sheet surface (49). In the next step, the formation of the thrombin-loaded tubular DNA nanorobots with additional anti-nucleolin aptamers at both ends is mediated by the addition of the fasteners and targeting strands. As mentioned before, this DNA nanorobot is programmed to open in response to exposure to nucleolin, which results in the release of its loaded thrombin (49). In conclusion, these DNA nanorobots can be considered immunologically inert drug delivery systems for cancer therapy.

3. Summary and Perspectives

The high-level expression of nucleolin by various cancer cells, as compared to normal cells, has made it an

Table 1. A Summary of Chemotherapeutic Drug Delivery Platforms Directed Against Nucleolin Using AS1411.

Delivery System	Components	Drug (s)	Animal Models or/and Cell Lines	Investigated Cancer Type	In Vivo / In Vitro	Reference
Nanomaterials						
Nanopolymersome	PEG-PLGA	Gemcitabine	A549	Non-small cell lung cancer	In vitro	(27)
Dendrimer	PEGylated PAMAM G5 dendrimer	Camptothecin	C26 tumor-bearing BALB/c mice / HT29, C26	Colon adenocarcinoma	In vivo / In vitro	(28)
Nanoparticle	PAMAM-PEG	5-fluorouracil	MKN45	Gastric cancer	In vitro	(29)
Nanoparticle	PGG	Paclitaxel	BALB/c nude mice / U87 MG	Glioblastoma	In vivo / In vitro	(19)
Dendrimer	ALGDG2	Iohexol	4t1 tumor mouse models / MCF-7	Breast cancer	In vivo / In vitro	(30)
Nanosphere	PLGA	Doxorubicin	BALB/c mice / C6	Glioma	In vivo / In vitro	(31)
Micelle	PEG-PDLLA	Triptolide	BALB/c nude mice / MIA PaCa-2	Pancreatic cancer	In vivo / In vitro	(32)
Nanoparticle	Mannitol-PLGA-TPGS	Docetaxel	Severe combined immunodeficient (SCID) mice / HeLa	Cervical cancer	In vivo / In vitro	(33)
Nanoparticle	HSA	5-fluorouracil, BpT	BALB/c nu/nu mice / Bel-7402	Hepatocellular carcinoma	In vivo / In vitro	(34)
Nanoparticle	Cholic acid-PLGA-*b*-TPGS	Docetaxel	Sprague-Dawley rats / MCF-7, MDA-MB-231	Breast cancer	In vivo / In vitro	(35)
Nanocluster	Gold nanoclusters (AuNC)-cRGD peptide MPA	Doxorubicin	Athymic nude mice / U87MG, MCF-7, L02, A549	Glioma, breast cancer, hepatic cancer, adenocarcinoma	In vivo / In vitro	(36)
Nanodroplet	DPPC-DSPE-PEG2000-DSPE-PEG2000-maleimide	Thymoquinone	MDA-MB-231, HCC1395	Breast cancer	In vitro	(37)
Dendrimer	ssDNA-based dendrimers	Epirubicin	BALB/c mice / MCF-7, C26	Breast cancer, colon carcinoma	In vivo / In vitro	(38)
Nanovesicle	PEP	Doxorubicin hydrochloride	BALB/c nude mice / MCF-7	Breast cancer	In vivo / In vitro	(39)
Nanoparticle	UnTHCPSi-PEI	Methotrexate, Sorafenib	MDA-MB-231	Breast cancer	In vitro	(20)
Nanoparticle	PEG-SPION/MMSNs	Doxorubicin	MCF-7	Breast cancer	In vitro	(40)
Nanocomposite	AuNP@$(AgNCs)_n$	-	HeLa	Cervical cancer	In vitro	(41)
Nanocomposite	UiO-66@AgNCs	Doxorubicin	MCF-7	Breast cancer	In vitro	(42)
Niosome	2,3-bis(tetradecyloxy)propan-1-aminium chloride, polysorbate 80	HoThyRu	HeLa	Cervical cancer	In vitro	(43)
Liposomes						
Liposome	DPPC-Cholesterol-PEG 2000-DSPE-ABC	Doxorubicin	BALB/c nude mice / MCF-7, ADR	Breast cancer	In vivo / In vitro	(22)
Liposome	Cholesterol/DSPE-PEG/HSPC	Doxorubicin	Nude mice / MCF-7	Breast cancer	In vivo / In vitro	(44)
Hydrogels						
Hydrogel	Acrydite-modified oligonucleotides, acrylamide	Doxorubicin	-	-	-	(25)

Abbreviations: PEG, polyethylene glycol; PLGA, poly(lactic-co-glycolic acid); PAMAM, polyamidoamine; PGG, poly (L-γ-glutamyl-glutamine); ALGDG2, anionic linear globular dendrimer generation 2; HAS, human serum albumin; PDLLA, poly(d, l-lactide); TPGS, tocopheryl polyethylene glycol 1000 succinate; MPA, A near infrared dye; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DSPE-PEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]; DSPE-PEG_{2000}-maleimide, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]; PEP, poly[methoxy-poly (ethylene glycol)/ethyl-p-aminobenzoate phosphazene]s; UnTHCPSi, Undecylenic acid modified, thermally hydrocarbonized porous silicon nanoparticles; PEI, polyethylenimine; SPION/MMSNs, superparamagnetic iron oxide nanoparticles/magnetic mesoporous silica nanoparticles; AuNP, gold nanoparticles; AgNC, silver nanoclusters; ABC, ammonium bicarbonate; BpT, 2-benzoylpyridine thiosemicarbazide copper II.

ideal prognostic marker and a promising target for cancer therapy with easy accessibility. So far, different anti-nucleolin agents that target different nucleolin domains have been investigated, each with different therapeutic effects. Among these different agents, the F3 peptide, anti-nucleolin pseudopeptides (named HB-19), anti-nucleolin antibodies, and the anti-nucleolin aptamer, AS1411, have been studied the most (50). As compared to anti-nucleolin antibodies and the F3 peptide, AS1411 has several advantages including resistance to nuclease activity, shorter generation time, higher tissue-penetration, lower manufacturing costs, higher modifiability, and better thermal stability. The AS1411 aptamer is also non-immunogenic, non-toxic, and very small, allowing its use in the production of biosensors for diagnostics.

As we comprehensively discussed throughout this review, aptamers, as non-immunogenic and non-toxic nucleotides, appear to be ideal candidates for redirecting the herein discussed drug delivery systems towards the desired tumor cells. We reviewed different studies regarding the use of the AS1411 aptamer for nucleolin-targeting delivery systems in a very efficient manner to deliver drugs, therapeutic agents, etc., which not only concentrate on elevating the therapeutic efficacy but also diminish the non-specific side effects of the mentioned approaches.

As discoursed in this article, it is proposed that the overexpression of nucleolin may provide a very worthwhile biomarker for a variety of tumors to be very more sensitive to being targeted by AS1411 (8). The ability of AS1411 to specifically target the external domain of surface nucleolin of cancer cells grants it a very novel tumor-selective behavior. To this day, this fact has been highlighted by many studies, which have demonstrated that AS1411 could be utilized as a drug delivery system and a very efficient target-

ing vehicle to deliver drugs, therapeutic agents, imaging probes, or nanoparticles to tumor cells (5).

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