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KE108-conjugated unimolecular micelles loaded with a novel HDAC inhibitor thailandepsin-A for targeted neuroendocrine cancer therapy

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ABSTRACT

Neuroendocrine (NE) cancers can cause significant patient morbidity. Besides surgery, there are no curative treatments for NE cancers and their metastases, emphasizing the need for the development of other forms of therapy. In this study, multifunctional unimolecular micelles were developed for targeted NE cancer therapy. The unimolecular micelles were formed by multi-arm star amphiphilic block copolymer poly(amidoamine)–poly(valerolactone)–poly(ethylene glycol) conjugated with KE108 peptide and Cy5 dye (abbreviated as PAMAM–PVL–PEG–KE108/Cy5). The unimolecular micelles with a spherical core–shell structure exhibited a uniform size distribution and excellent stability. The hydrophobic drug thailandepsin-A (TDP-A), a recently discovered HDAC inhibitor, was physically encapsulated into the hydrophobic core of the micelles. KE108 peptide, a somatostatin analog possessing high affinity for all five subtypes of somatostatin receptors (SSTR 1–5), commonly overexpressed in NE cancer cells, was used for the first time as an NE cancer targeting ligand. KE108 exhibited superior targeting abilities compared to other common somatostatin analogs, such as octreotide, in NE cancer cell lines. The *in vitro* assays demonstrated that the TDP-A-loaded, KE108-targeted micelles exhibited the best capabilities in suppressing NE cancer cell growth. Moreover, the *in vivo* near-infrared fluorescence imaging on NE-tumor-bearing nude mice showed that KE108-conjugated micelles exhibited the greatest tumor accumulation due to their passive targeting and active targeting capabilities. Finally, TDP-A-loaded and KE108-conjugated micelles possessed the best anticancer efficacy without detectable systemic toxicity. Thus, these novel TDP-A-loaded and KE108-conjugated unimolecular micelles offer a promising approach for targeted NE cancer therapy.

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1. Introduction

Neuroendocrine (NE) cancers are the second most prevalent gastrointestinal tract malignancy after colorectal cancer [1–4]. Surgical resection is the only curative option, but most patients are not candidates for operative intervention due to widespread metastases at the time of presentation [5,6]. Moreover, other forms of therapy have had limited efficacy, emphasizing the need for the development of new therapy [7].

We have an ongoing interest in identifying effective approaches of reactivating tumor suppressor pathways and altering malignant NE phenotypes. In previous studies, we showed that the inhibition of histone deacetylases (HDACs) triggered activation of Notch signaling and induced cell cycle arrest and apoptosis, leading to NE cancer cell death [8–11]. HDAC inhibitors have hence emerged as a new type of anticancer agent. Up to now, three HDAC

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inhibitors—vorinostat (SAHA, Zolinza®) [12], belinostat (PXD-101, Beleodaq®) [13], and natural product FK228 (romidepsin, Istodax®) [14]—have been approved by the FDA. Thailandepsin A (TDP-A), a natural and potent HDAC inhibitor, was recently isolated by Cheng [15] from the bacterium Burkholderia thailandensis E264. TDP-A can selectively and strongly inhibit class I HDACs including HDAC1, HDAC2 and HDAC3 [15,16]. It also showed potent cytotoxic activities against NE and endocrine cancer cell lines at low nanomolar concentrations [11,17]. Its high potency and selective inhibition of class I HDACs makes TDP-A a promising candidate as an HDAC inhibitor. However, TDP-A is hydrophobic and lacks *in vivo* tumor-targeting capability when administered systemically, leading to dose-limiting toxicity and thereby limiting its application.

To overcome the poor water solubility, improve the biostability, and increase the tumor accumulation of TDP-A, we have developed a unique unimolecular micelle nanoparticle as a nanocarrier for TDP-A. Drug nanocarriers can not only improve the water solubility and stability of hydrophobic drugs, but also increase the therapeutic index of drugs by delivering them specifically to tumor sites via the enhanced permeability and retention (EPR) effect [18,19]. Moreover, drug nanocarriers conjugated with specific tumor-targeting ligands (e.g., peptides, antibodies, aptamers, or small molecules) can further increase their accumulation at target sites and therefore significantly enhance a drug's therapeutic effects [20–23].

Polymer micelles self-assembled from linear amphiphilic block copolymers have been widely studied as drug carriers [24–28]. Polymer micelles exhibit a core–shell architecture where the hydrophobic core serves as a natural carrier environment for hydrophobic drugs, while the hydrophilic shell imparts the micelle nanoparticle aqueous solubility. However, one major concern with the most widely studied drug delivery nanocarriers—including polymer micelles and vesicles, as well as liposomes, which are formed via the self-assembly of a large number of linear amphiphilic block copolymers or phospholipids—is their *in vivo* stability due to the dynamic nature of the self-assembly process. The *in vivo* stability of these self-assembled drug nanocarriers is affected by a variety of factors, including the concentration of the amphiphilic molecules, pH, temperature, ionic strength, or their interaction with proteins [29–31]. Premature dissociation of self-assembled drug nanocarriers during circulation in the bloodstream can cause a burst release of anticancer drugs into the bloodstream, which not only leads to potential systemic toxicity, but also undermines their tumor-targeting ability, thereby severely limiting their use for *in vivo* applications. Hence, there is a need to develop strategies to enhance the *in vivo* stability of self-assembled drug nanocarriers. Unimolecular micelles formed by individual/single multi-arm star amphiphilic block copolymer molecules exhibit excellent stability *in vitro* and *in vivo* due to their unique chemical structure and covalent nature [32–40]. These unique unimolecular micelles have been successfully used to deliver various compounds to tumor tissues in a targeted manner [32–40].

The majority of NE cancers overexpress somatostatin receptors (SSTRs, a family of guanosine triphosphate-binding protein-coupled receptors, of which there are five subtypes, SSTR 1–5) [37,41–43]. Our previous studies, as well as others, have shown that octreotide, a somatostatin analog with a strong binding affinity to SSTR, can be used as an effective tumor-targeting ligand for targeted NE cancer therapy [37,41]. Octreotide displays a high binding affinity to SSTR2 and a moderate affinity to SSTR5 [44]. It was the first somatostatin analog used in clinic, but has very little affinity to the other three SSTR subtypes (i.e., SSTR1, SSTR3, and SSTR4) [44–46]. On the other hand, KE108, another somatostatin analog, shows a pan-somatostatin profile because it binds to all five SSTRs with high affinity (Fig. 1 (A)) [47], thus making it a superior targeting ligand for SSTRs. Despite its strong potential as an effective active NE tumor-targeting ligand, there have not been any reports on KE108-conjugated drug nanocarriers.

In this study, for the first time, a multifunctional unimolecular micelle nanoplatform using **KE108** as the active tumor-targeting ligand was developed for targeted NE cancer drug delivery (e.g., TDP-A). The unimolecular micelles were formed by multi-arm star amphiphilic block copolymer poly(amidoamine)–poly(-velarolactone)–poly(ethylene glycol) conjugated with KE108 peptide and Cy5 dye (abbreviated as PAMAM–PVL–PEG–KE108/Cy5) (Fig. 1 (B)). Cy5 dye was conjugated for visualization and detection of micelles both *in vitro* and *in vivo*. TDP-A was encapsulated inside of the hydrophobic core of the unimolecular micelles through hydrophobic interactions and hydrogen bonding. These drug-loaded unimolecular micelles are capable of both passive and active targeting for NE cancer therapy as illustrated in Fig. 1 (C).

2. Methods

2.1. Materials

Poly(amidoamine) (1,4-diaminobutane; G4) dendrimer was purchased from NanoSynthons LLC (Mt. Pleasant, MI, USA). Dimethyl sulfoxide (DMSO), velarolactone (VL), and stannous (II) octoate ($Sn(Oct)_2$) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The heterobifunctional poly(ethylene glycol) (PEG) derivatives, methoxy–PEG–OH (mPEG–OH, M_n = 5 kDa) and OH–PEG–*N*–hydroxysuccinimide (HO–PEG–NHS, M_n = 5 kDa) were purchased from JenKem Technology (Allen, TX, USA). 4-Dimethylamino pyridine (DMAP) and 1,3-dicyclohexylcarbodiimide (DCC) were purchased from ACROS and used without further purification. KE108 was purchased from Bachem Americas, Inc. (Torrance, CA, USA). Cy5 dye was obtained from Lumiprobe Corporation (Hallandale Beach, FL, USA). Other reagents were purchased from Thermo Fisher Scientific (Fitchburg, WI, USA) and used as received unless otherwise stated.

2.2. Synthesis of PAMAM–PVL–OH

PAMAM–PVL–OH was prepared by ring-opening polymerization of the VL monomer using PAMAM–OH as the macro-initiator (Scheme 1). A 50 ml two-neck flask equipped with an argon gas inlet was charged with PAMAM–OH (20 mg, 1.4 μmol) and placed in an oil bath. VL (277 mg, 2.7 mmol) and a catalytic amount of $Sn(Oct)_2$ ($[Sn(Oct)_2]/[VL]$ = 1/1000 mol/mol) was added subsequently. The reaction was carried out at 120 °C for 24 h. The resulting mixture was dissolved in THF and the solution was added dropwise into methanol to yield a pale yellow precipitate. The final product, PAMAMA–PVL–OH, was dried under vacuum.

2.3. Synthesis of PAMAM–PVL–COOH

PAMAM–PVL–COOH was prepared by a reaction between PAMAM–PVL–OH (50 mg, 0.51 μmol) and succinic anhydride (8.2 mg, 82 μmol) in the presence of DMAP (12.5 mg, 98 μmol). The reaction was carried out in anhydrous DCM (10 mL) for 48 h at room temperature. Thereafter, the resulting solution was added into 10 fold of cold diethyl ether. The precipitate was redispersed in DI water and the impurities were removed by dialysis against DI water using cellulose tubing (molecular weight cut-off, 15 kDa) for 48 h. The final product was obtained after lyophilization.

2.4. Synthesis of PAMAM–PVL–PEG–OCH_3/NHS

PAMAM–PVL–PEG–OCH_3/NHS was synthesized by reacting

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