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Targeting NCAM-expressing neuroblastoma with polymeric precision nanomedicine

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ABSTRACT

Neural cell adhesion molecule (NCAM) expression is known to be associated with an aggressive biological behavior, increased metastatic capacity and expression of stem-cell markers in several tumor types. NCAM was also found to be expressed on tumor endothelial cells while forming new capillary-like tubes, but not on normal endothelial cells. An NCAM-targeted polymer-drug conjugate can be used both to target tumors expressing high levels of NCAM as well as the angiogenic vessels and cancer stem cells populations characterized by NCAM expression within tumors. Here, we describe the design, synthesis, physico-chemical characterization and the biological evaluation of an NCAM-targeted conjugate of polyglutamic acid with paclitaxel that was developed and evaluated on neuroblastoma, a high NCAM-expressing tumor. This conjugate inhibited tumor growth to a higher extent compared to the control conjugates and was less toxic than free paclitaxel. The dose of the conjugate could be increased at least twice than the maximum tolerated dose of paclitaxel to achieve better activity without aggravating toxicity. This work presents evidence that NCAM targeting can highly increase the efficacy of nanomedicines in the appropriate tumor models.

1. Introduction

Neuroblastoma is a tumor derived from primitive cells of the sympathetic nervous system and is the most common solid tumor in childhood. About half of all neuroblastomas arise in the adrenal medulla, and the rest originate in paraspinal sympathetic ganglia in the chest or abdomen, or in pelvic ganglia. Neuroblastomas account for 7–10% of all childhood cancers, and it is the most common cancer diagnosed during infancy [1].

Neural cell adhesion molecule (NCAM/CD56) is a cell adhesion molecule structurally belonging to the immunoglobulin superfamily. NCAM is overexpressed on many tumor types, such as melanoma, glioblastoma, neuroblastoma and others [2–8]. NCAM expression is known to be associated with more aggressive biological behavior, increased metastatic capacity, expression of stem-cell markers and poor prognosis in several tumor types [6,7,9–15]. In neuroblastoma, high expression of NCAM is frequently associated with cancer progression and inhibition of tumor cell adhesion to the endothelium [7]. Interestingly, NCAM was found to be expressed on tumor endothelial cells, but not on normal endothelial cells [16]. However, in other tumors, such as in advanced glioma, the loss of NCAM correlates with a more aggressive tumor phenotype and with poor prognosis [17]. We chose to work on neuroblastoma, which exhibits high expression of NCAM that is frequently associated with cancer progression [12,18–20]. Specifically, IMR-32 human neuroblastoma cell line was selected since it exhibits MYCN amplification, the hallmark of aggressive neuroblastoma, and is known to form highly angiogenic tumors when injected orthotopically into the adrenal gland [21–23].

NCAM exists in three major isoforms; a 120 kDa isoform connected by a GPI (glycosyl-phosphatidylinositol) anchor to the cell membrane, and 140 and 180 kDa isoforms, which contain a transmembrane domain [24]. The identification of specific NCAM isoforms in different malignancies has been the focus of several studies. It was found that the embryonic, transmembrane 140 and 180 kDa isoforms are most often associated with disease progression and malignant potential [5,15,25,26]. The 120 kDa isoform was also found to be expressed in neuroblastoma [27]. We designed an NCAM-targeted conjugate for the selective

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binding to all NCAM isoforms since it binds only to the extracellular domain, which is common to all three isoforms.

In order to use NCAM as a relatively selective target for drug delivery systems to the tumor tissue, a suitable polymeric carrier was required. On that end, an attractive polymeric carrier which is water-soluble, non-toxic and multivalent polymer was polyglutamic acid (PGA) [28–30]. It is composed of units of naturally occurring L-glutamic acid linked together through amide bonds. The pendant free γ-carboxyl group in each repeating unit of L-glutamic acid is negatively charged at a neutral pH, which renders the polymer water-soluble. The carboxyl groups also provide functionality for drug attachment. PGA is enzymatically biodegradable by cathepsin B, highly expressed in most tumor tissues [31–34]. It is non-immunogenic and enables multivalent binding of drugs and targeting moieties. Hence, when used at an appropriate nanoscaled size, PGA conjugate can allow selective extravasation-dependent delivery to tumors *via* the leaky angiogenic tumor vessels. Indeed, the most clinically-advanced polymer-drug conjugate is PGA-paclitaxel (PGA-PTX) (OPAXIO™). It is currently being evaluated in Phase III clinical trials for ovarian cancer as a single agent [35,36], and for non-small-cell lung cancer in combination with carboplatin [37]. A Phase III clinical trial was recently concluded for OPAXIO™ in combination with temozolomide and radiotherapy for the treatment of glioblastoma, showing OPAXIO™ may enhance the therapeutic effect of radiation and increase progression-free and overall survival [38].

C3 peptide, a known NCAM agonist [39–41], was selected as a targeting moiety defined here as NCAM Targeting Peptide (NTP). It binds to Ig1 domain of NCAM and was also found to bind to fibroblast growth factor receptor 1 (FGFR1) [42]. The ability of C3 peptide to target NCAM-expressing cancer cells was demonstrated on Kaposi's sarcoma using doxorubicin (DOX)-loaded liposomes [43]. We propose here to rationally-design an NCAM-targeted PGA-PTX nanomedicine for NCAM-expressing tumors in general and for neuroblastoma, in particular.

2. Materials and methods

2.1. Ethics statement

Animal procedures were in compliance with and approved by the Institutional Animal Care and Use Committee (IACUC) of Tel Aviv University.

2.2. Materials

Paclitaxel was obtained from Petrus Chemicals and Materials Ltd. (Israel). PGA was obtained from Alamanda Polymers (Huntsville, AL, USA) or synthesized in our laboratory according to published protocols [44]. All other chemical reagents were obtained from Merck or Sigma-Aldrich, unless otherwise stated. HPLC grade solvents were obtained from Biolab, Israel. Tissue culture reagents were obtained from Biological Industries Ltd., Israel, unless indicated otherwise.

2.3. NCAM targeting peptide (NTP) synthesis

NCAM targeting peptide with the sequence GASKKPKRNIKA (C3 peptide - NTP) and a control peptide with the sequence GASKKPAANIKA (C3ala peptide - cNTP) were synthesized using solid phase peptide synthesis (SPPS) method on Sieber amide resin. Glycine was added at the N-terminal as a linker to allow conjugation to PGA or fluorescent labeling. Molecular mass of the products was confirmed by mass spectroscopy (MS) to match the calculated mass of peptides, indicating that the correct compounds were obtained (Supplementary Fig. 1A). To obtain fluorescently-labeled peptides, 5(6)-carboxyfluorescein was coupled to the N-terminal of the peptides on resin. Its molecular mass was also confirmed by MS (Supplementary Fig. 1B).

Purity of the peptides was evaluated using HPLC. A single peak was obtained, indicating the product is pure (Supplementary Fig. 2).

2.4. Synthesis of PGA-PTX

PGA-PTX control conjugate was synthesized as previously published [45]. Briefly, PTX (29.7 mg, 34.8 μmol) was conjugated to PGA (150.0 mg, 1.16 mmol; 100 units, Mw ~ 13 kDa) by carbodiimide coupling (*N*,*N*-diisopropylcarbodiimide (DIC), 1-hydroxybenzotriazol (HOBt) and *N*,*N*-dimethylaminopyridine (DMAP) were used as coupling reagents and diisopropylethylamine (DIPEA) was added in excess as a base) in anhydrous DMF (10 mL) under N_2. After 24 h, the solvent was evaporated under high vacuum and the residue washed in chloroform:acetone (4:1). The resulting precipitate was washed in chloroform:acetone (2:2) and dried under vacuum to obtain the PGA-PTX conjugate. The supernatant of the chloroform:acetone washing mixture was kept to determine drug loading by measuring the amount of unreacted PTX by analytical HPLC. The water-soluble sodium salt of the conjugate was obtained by dissolving the product in 0.25 M $NaHCO_3$ after evaporation of DMF. This aqueous solution was purified by SEC using Sephacryl S-200 HR column (GE Healthcare, Buckinghamshire, UK), removing unreacted drugs and low molecular weight contaminants and lyophilized to obtain the final product as a white powder (elution fractions 85–160 mL).

2.5. Synthesis of PGA-PTX-NTP

For the conjugation of PTX and NTP to PGA, PTX was bound directly to the PGA by an ester bond as described above and the NTP peptide was bound by an amide bond at the N-terminal of the peptide. PGA-PTX-NTP conjugate was synthesized using the following steps (Scheme 1).

2.5.1. PGA-NTP synthesis

NTP peptide was cleaved from the resin using a mild cleavage process that leaves protecting groups on the side chains of amino groups intact. This was done to achieve selective coupling to the PGA through the N-terminal amine of the peptide and not through amines in the peptide sequence (lysines). The protected peptide (104 mg, 46.5 μmol) was conjugated to PGA (200 mg, 1.55 mmol) using carbodiimide coupling (DIC/HOBt and DMAP were used as coupling reagents and diisopropylethylamine (DIPEA) was added in excess as a base) in dry DMF under N_2 followed by deprotection of the peptide side chains. Deprotection was done with a mixture of 95% TFA 2.5% DDW, 2.5% triisopropylsilane for 4 h. The solvent was evaporated until ~1 mL remained and cold ether was added to precipitate the PGA-NTP conjugate. The product was washed with cold ether and dried under vacuum.

2.5.2. PGA-PTX-NTP synthesis

PTX (200.0 mg, 232 μmol) was conjugated to PGA-NTP (230.0 mg, 1.55 mmol) by carbodiimide coupling (DIC/HOBt and DMAP were used as coupling reagents and DIPEA was added in equivalent amount to PTX moles as a base) in anhydrous DMF (10 mL) under N_2. While conjugating PTX to PGA-NTP, the lysine groups on the NTP were protonated after deprotection with TFA and were therefore not reactive. The amount of DIPEA had to be carefully controlled so that it would be sufficient for conjugation but would not deprotonate the amine groups of the lysines. The PGA-PTX-NTP conjugate was isolated and purified as described above for PGA-PTX.

PGA-PTX-cNTP control conjugate was synthesized by the same method.

2.5.3. FITC labeling of the conjugates

To obtain fluorescently-labeled conjugates, PGA conjugates were incubated with fluorescein isothiocyanate (FITC) in borate buffer at pH = 9 for 4 h. The conjugate was purified using a Sephacryl S-200 HR column and the FITC-labeled fraction was collected and lyophilized (Supplementary Fig. 3).