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Polymalic acid nanobioconjugate for simultaneous inhibition of tumor growth and immunostimulation in HER2/*neu*-positive breast cancer[☆]

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Abstract

Breast cancer remains the second leading cause of cancer death among women in the United States. The breast cancer prognosis is particularly poor in case of tumors overexpressing the oncoprotein HER2/*neu*. A new nanobioconjugate of the Polycefin family of anti-cancer drugs based on biodegradable and non-toxic polymalic acid (PMLA) was engineered for a multi-pronged attack on HER2/*neu*-positive breast cancer cells. An antibody cytokine fusion protein consisting of the immunostimulatory cytokine interleukin-2 (IL-2) genetically fused to an antibody specific for human HER2/*neu* [anti-HER2/*neu* IgG3-(IL-2)] was covalently attached to the PMLA backbone to target HER2/*neu* expressing tumors and ensuring the delivery of IL-2 to the tumor microenvironment. Antisense oligonucleotides (AON) were conjugated to the nanodrug to inhibit the expression of vascular tumor protein laminin-411 in order to block tumor angiogenesis. It is shown that the nanobioconjugate was capable of specifically binding human HER2/*neu* and retaining the biological activity of IL-2. We also showed the uptake of the nanobioconjugate by HER2/*neu*-positive breast cancer cells and enhanced tumor targeting *in vivo*. In addition, the nanobioconjugate was capable of eliciting anti-tumor activity in immunocompetent mice bearing

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D2F2/E2 murine mammary tumors that express human HER2/*neu*. Both IgG1 and IgG2a levels were significantly increased in animals treated with the PMLA–fusion nanobioconjugate compared to animals treated with the antibody–cytokine fusion protein alone or control animals, indicative of the induction of a humoral (T_H2) and cell-mediated (T_H1) immune responses. Animal survival *in vivo* was significantly longer after treatment with leading nanobioconjugate with fusion [anti-HER2/*neu* IgG3-(IL-2)] antibody, $p < 0.05$. The combination of these molecules on a single polymeric platform is expected to act through direct elimination of cancer cells, inhibition of tumor angiogenesis, and orchestration of a potent immune response against tumor.

Keywords

Polymalic acid; Nanobioconjugate; Nanopolymer; HER2/*neu*; Antibody fusion protein; IL-2; Laminin-411; Breast cancer

1. Introduction

According to the American Cancer Society, in 2013 an estimated 232,340 new cases of invasive breast cancer will be diagnosed among women and approximately 39,620 women are expected to die from breast cancer [1]. Although only 25–30% of human breast and ovarian tumors overexpress the HER2/*neu* (epidermal growth factor receptor 2) proto-oncogene, the elevated level of HER2/*neu* in malignancies correlates strongly with poor prognosis [2–5]. Its extracellular accessibility makes it an excellent target for tumor-specific therapeutic agents. In fact, using the FDA-approved anti-HER2/*neu* antibody trastuzumab (Herceptin®) alone or combined with chemotherapy in patients with advanced breast cancer leads to an objective response against tumors overexpressing HER2/*neu* [6–11]. However, a significant number of these patients do not respond to trastuzumab-based treatments and most that do initially respond acquire resistance within one year and eventually succumb to the disease [12–15]. Therefore, strategies for improved treatment of HER2/*neu* expressing cancers are still needed.

Interleukin-2 (IL-2) is a potent immunostimulator of both cellular and humoral adaptive immune responses and has been studied extensively as a potential cancer treatment [16,17]. It primarily not only stimulates T-cells to proliferate and become cytotoxic, but also activates a variety of immune cells including NK cells, lymphokine-activated killer (LAK) cells, monocytes, and macrophages [16]. Given the rapid blood clearance of IL-2 and its lack of tumor specificity, its therapeutic potential is limited since systemic administration of IL-2 in high doses is associated with severe toxicities, including vascular leak syndrome [17–19]. Therefore, our goal was to target sufficient quantities of IL-2 to the site of HER2/*neu*-expressing tumors using an antibody–cytokine fusion protein [20,21]. We previously developed an antibody–cytokine fusion protein that consists of human IL-2 genetically fused to the C-terminus of each heavy chain of an anti-HER2/*neu* IgG3 [anti-HER2/*neu* IgG3-(IL-2)] [22]. We used human IL-2 since it is fully active in mice and human IgG3 due to its extended hinge region that confers spacing and flexibility [23]. Compared to other IgG isotypes (IgG1, IgG2, and IgG4) IgG3 is the most flexible [23]. We consider the extended hinge of human IgG3 of particular relevance in the context of its biorecognition and biological activity, since it would minimize steric hindrance and further facilitate binding to the antigen and cytokine receptor. This molecule was developed to enhance the tumoricidal activity of the antibody alone as well as elicit tumor specific immune responses due to the delivery of IL-2 to the tumor microenvironment. The extracellular domain of HER2/*neu* (ECD^{HER2}) is insufficiently processed by dendritic cells and thus is poorly immunogenic [24]. Using immunostimulators such as IL-2 is expected to enhance the immunogenicity of HER2/*neu*. In fact, we have shown that the antibody–cytokine fusion protein is very

efficiently internalized by dendritic cells and trafficked to antigen-processing compartments [25]. Importantly, the anti-HER2/*neu* IgG3-(IL-2) acts as a direct anti-cancer agent [22,26] as well as an adjuvant of ECD^{HER2} vaccination [25,27–29] in murine models under conditions where the antibody alone fails to confer protection. In all of our previous studies the anti-HER2/*neu* IgG3-(IL-2) was well-tolerated and no unexpected side-effects were observed in treated animals.

Combination therapy is a common practice for the treatment of breast cancer [30,31] and it may greatly improve patient survival using a strategy designed to combine immunotherapy with nanoparticles specifically targeting the tumor microenvironment [32]. Laminin-411 (formerly, laminin-8) and its integrin receptors $\alpha 6\beta 1$ and $\alpha 5\beta 1$ are important for the functioning of endothelial cell basement membranes [33,34]. It promotes cell migration during development, wound healing, and angiogenesis *in vivo* and *in vitro* [34–36]. We identified laminin-411 as a critical angiogenic marker of the brain and breast cancer [37–40]. Using poly(β -l-malic acid) (PMLA)-based nanobioconjugates for targeted delivery of antisense oligonucleotides (AON) blocking the synthesis of laminin-411 chains indeed inhibited tumor cell migration and invasion [39,41].

PMLA, a natural product of the slime mold *Physarum polycephalum* [42–44], is a promising drug carrier well developed by our group. The attractive properties of PMLA as a carrier matrix for biopharmaceuticals are its lack of toxicity *in vitro* and *in vivo*, non-immunogenicity, biodegradability, and versatility for drug loading. In this study, a PMLA nanobioconjugate was rationally designed for combination therapy to simultaneously deliver anti-HER2/*neu* IgG3-(IL-2) and AON against laminin-411 chains specifically to the tumor site in order to achieve increased therapeutic efficacy and reduce adverse effects to normal tissues. The nanobioconjugate based on PMLA as a drug carrier contains 40% leucine ethyl ester as an endosomal escape unit, anti-HER2/*neu* IgG3-(IL-2) for tumor targeting and anti-tumor immunotherapy, and Morpholino AON against laminin-411 $\alpha 4$ and $\beta 1$ chains to inhibit tumor angiogenesis. The designed nanobioconjugate aims to reduce the toxicities of the cytokine, to improve the circulation time of the nanobioconjugate, and to inhibit breast cancer growth more efficaciously than anti-HER2/*neu* IgG3-(IL-2) alone.

2. Materials and methods

2.1. Materials

Highly purified, endotoxin-free PMLA, with a weight-averaged molecular weight (mw) of 60 kDa and a polydispersity of 1.1, was obtained from the culture broth of *P. polycephalum* and size-fractionated on Sephadex G25 (GE Healthcare, Pittsburgh, PA). Cysteamine (2-mercaptoethylamine hydrochloride), N-hydroxysuccinimide, and other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Morpholino-3'-NH₂ AON AGC-TCA-AAG-CCA-TTT-CTC-CGC-TGA-C to laminin $\alpha 4$ and CTA-GCA-ACT-GGA-GAAGCC-CCA-TGC-C to laminin $\beta 1$ chains were custom made by Gene Tools (Philomath, OR). mPEG₅₀₀₀-amine and maleimide-PEG₃₄₀₀-maleimide were obtained from Laysan Bio, Inc. (Arab, AL). Alexa Fluor 680 C₂ maleimide and Alexa Fluor 488 C₅ maleimide were from Invitrogen (Carlsbad, CA).

2.2. Cell lines and culture conditions

Two human HER2/*neu*-positive breast cancer cell lines SK-BR-3 and BT-474 were obtained from American Type Culture Collection (Manassas, VA). BT-474 was cultured in DMEM with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and antibiotics. SK-BR-3 was cultured in RPMI-1640 medium with 10% FBS and antibiotics. The D2F2 mouse mammary tumor cell line (syngeneic to BALB/c mice) and that transfected with a

vector encoding human HER2/*neu* (D2F2/E2) were kind gifts from Dr. Wei-Zen Wei (Wayne State University, Detroit, MI) [45] and were cultured in IMDM (Invitrogen) containing 10% FBS. Growth media for D2F2/E2 was further supplemented with 1 mg/ml G418 (a selective antibiotic for the neomycin resistance gene) to maintain high expression of human HER2/*neu*.

2.3. Production of the antibody–cytokine fusion protein, anti-HER2/*neu* IgG3-(IL-2)

To improve the efficacy of the humanized antibody trastuzumab, we have genetically fused the IL-2 cytokine to a human IgG3 with the variable regions of trastuzumab (Herceptin®). The scheme of genes encoding the heavy chain of the antibody–cytokine fusion protein is depicted in Fig. 1A and a schematic representation of fusion anti-HER2/*neu* IgG3-(IL-2) is depicted in Fig. 1B. Murine myeloma cells (P3X63-Ag8.653) expressing and secreting the antibody–cytokine fusion protein were grown in roller bottles and purified from cell culture supernatants using affinity chromatography as previously described [22,46]. Protein concentration was determined by bicinchoninic acid-based protein assay (BCA Protein Assay, Thermo Fisher Scientific Inc., Walnut Creek, CA). Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The parental antibody without IL-2, anti-HER2/*neu* IgG3, has been previously described and used as a control in studies with the fusion protein [22,25–29]. In figures anti-HER2/*neu* IgG3 and anti-HER2/*neu* IgG3-(IL-2) are abbreviated [IgG3 and IgG3-(IL-2)] due to space constraints.

2.4. Synthesis of PMLA-fusion nanobioconjugate, P/mPEG/LOEt/IgG3-(IL-2)/AON(α 4 β 1)

The copolymer PMLA/mPEG/LOEt/MEA containing 5% mPEG, 40% leucine ethyl ester (LOEt), and 10% 2-mercaptoethylamine (MEA) was prepared as previously described (% referring to the total amount of pendant carboxyl groups in PMLA) [41,47], where mPEG increases the stability of nanobioconjugate in bloodstream, LOEt serves as an endosomal escape unit to achieve cytoplasmic delivery, and MEA provides sufficient sulfhydryl groups for antibody conjugation, AON loading, and fluorescence labeling. The copolymer was conjugated to the antibody–cytokine fusion protein through a flexible spacer PEG₃₄₀₀ in an attempt to retain its biological activity [41,47]. The loading of antibody is 0.25% to ensure each polymer chain has one antibody–cytokine fusion protein for biorecognition and immunotherapy. After 3'-modification with 2-pyridyldithiopropionate, α 4 and β 1 AONs (designed to knock down the expression of both human and mouse laminin-411) were conjugated to the PMLA backbone via disulfide bond formation, which is cleavable in cytoplasm by glutathione after the nanobioconjugate escapes from endosome [41,47]. Unreacted sulfhydryl groups on PMLA backbone were blocked with 2-pyridyldithiopropionate. The nanobioconjugate was purified with Sephadex G-75 column (GE Healthcare) using PBS as the solvent. Alternatively, nanobioconjugates that were used for imaging were fluorescently labeled with Alexa Fluor 680 C₂ maleimide or Alexa Fluor 488 C₅ maleimide by forming thioether with sulfhydryl groups, respectively. A schematic representation of the new PMLA nanobioconjugate is shown in Fig. 1C. The synthesis of the nanobioconjugate was monitored by size exclusion high-performance liquid chromatography (HPLC). The total amount of malic acid was determined with malate dehydrogenase assay [48]. The total amount of loaded fusion antibody was estimated with MicroBCA protein assay (Thermo Fisher Scientific). A colorimetric assay was used to determine the loading of mPEG as previously described [49]. The loaded amount of AON was estimated with reverse phase HPLC after reductive cleavage of AON from the nanobioconjugate using known concentrations of AON as standard. The entire P/mPEG/LOEt/IgG3-(IL-2)/AON(α 4 β 1) nanobioconjugate is referred to as the lead PMLA-fusion nanobioconjugate.

2.5. Detection of HER2/neu and laminin- α 4 and β 1 expressions by western blot

Western blot analysis was performed as previously described [41,50]. Proteins were detected using the following primary monoclonal antibodies: mouse anti-laminin α 4 (clone 8F12; a gift from Dr. Sekiguchi, Department of Biological Sciences, Osaka University, Osaka, Japan), rat anti-laminin β 1 (Abcam, Cambridge, MA) and mouse anti-human HER2/neu (Cell Signaling Technology, Inc., Danvers, MA). Rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling Technology, Inc.) was used as a loading control. All four primary antibodies cross-react with both mouse and human proteins. The Immune-Star chemiluminescent kit (Bio-Rad Laboratories, Inc., Hercules, CA) was used as the substrate.

2.6. HER2/neu binding of the PMLA-fusion nanobioconjugate (flow cytometry)

Two hundred fifty thousand D2F2/E2 murine mammary cancer cells expressing human HER2/neu were incubated at 4 °C for 4 h in the presence of 2 μ g/ml of PMLA-fusion nanobioconjugate or the equivalent amount of anti-HER2/neu IgG3-(IL-2) or anti-HER2/neu IgG3 followed by a 1-hour incubation with rabbit anti-human κ antibody conjugated to FITC (BD Biosciences, San Jose, CA). Samples were analyzed on a Becton Dickinson FACScan™ Flow Cytometer in the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility. Histograms were created using the FCS Express V3 software (De Novo Software, Los Angeles, CA).

2.7. IL-2 activity (proliferation assays)

Murine CTLL-2 T-cells that are dependent on IL-2 for growth, were cultured in RPMI 1640 (Invitrogen) supplemented with 100 U/ml penicillin, 10 μ g/ml streptomycin, and 10% and 10 U/ml of recombinant human IL-2 (PeproTech, Rocky Hill, NJ) for five days. After washing five times with RPMI 1640, cells were incubated in 96-well plates (2×10^4 cells/well) for 18 h at 37 °C, 5% CO₂ in the presence of an equivalent molar amount of PMLA-fusion nanobioconjugate, anti-HER2/neu IgG3, or anti-HER2/neu IgG3-(IL-2), serially diluted 3-fold over a range from 0.13 to 30 ng/ml. Proliferation was measured by [³H]-thymidine incorporation assay as previously described [51]. Cells were treated in quadruplicate for 18 h followed by a 6-hour pulse with [³H]-thymidine (MP Biomedicals, Solon, OH).

2.8. In vitro cellular internalization (confocal microscopy)

The human breast cancer cell line BT-474 overexpressing human HER2/neu was incubated in the presence of Alexa Fluor 488-labeled PMLA nanobioconjugate with or without fusion anti-HER2/neu IgG3-(IL-2) (maximum excitation/emission wavelength: 488/519 nm). After incubation for 2 min, 30 min, and 1 h, the cells were washed with PBS and fixed with 4% paraformaldehyde. A TCS SP spectral scanner (Leica Microsystems, Mannheim, Germany) was used for confocal microscopy.

2.9. In vivo biodistribution of PMLA-fusion nanobioconjugate

One million BT-474 human breast cancer cells were implanted into the right flank of athymic mice (CrTac:NCr-Foxn1nu Homozygous, Taconic, Hudson, NY). When tumors reached 120 mm³ (about 21 days after tumor implantation), 150 μ l of Alexa Fluor 680 labeled PMLA copolymer, antibody-cytokine fusion protein or the PMLA-fusion nanobioconjugate was injected intravenously (i.v.) at the concentration of 10 μ M Alexa Fluor 680. For assessment of drug distribution and localization in nude mice, Xenogen IVIS 200 (STTARR, Toronto, ON, Canada) was used under isoflurane anesthesia at different time points (before drug administration, 1, 3, 6, and 24 h after injection of the drug). Twenty-four hours after drug administration, the mice were euthanized and the circulating drugs in blood

vessels were eliminated by intra-arterial PBS perfusion. Tumors were harvested to detect the fluorescent signal. The fluorescent signal intensities in the tumors were analyzed by Xenogen Living Image®, Version 2.50. All animal procedures were carried out in accordance with IACUC protocols approved by animal welfare committees at Cedars-Sinai Medical Center and UCLA.

2.10. In vivo efficacy studies in a syngeneic breast cancer model

D2F2/E2 cells were harvested using 0.5 mM EDTA in PBS and resuspended in Hank's balance salt solution (HBSS). Immunocompetent BALB/c mice were injected subcutaneously (s.c.) with 10^6 tumor cells on day 0. On days 6 and 8, mice were systemically treated by i.v. injection with either 10 μ g of anti-HER2/*neu* IgG3-(IL-2) or the molar equivalent amount of PMLA-fusion nanobioconjugate. Control animals were injected with buffer (PBS). Tumor growth was monitored with a caliper and the volume calculated using the formula: tumor volume = (length \times width ²) / 2 [52], and survival considered as the period of time from tumor challenge until the tumor diameter reaches 1.5 cm when mice were euthanized.

2.11. Assessment of murine anti-(ECD^{HER2}) antibodies in serum

Blood samples from animals in the *in vivo* efficacy studies were taken on day 27. The level of murine anti-(ECD^{HER2}) IgG1 and IgG2a was determined in pooled serum as described previously [29].

3. Results

3.1. Characteristics of PMLA-fusion nanobioconjugate

The PMLA-fusion nanobioconjugate (Fig. 1C) contains multiple components for the combination therapy of anti-angiogenesis and immunotherapy for breast cancer treatment. PMLA is biodegradable and nontoxic (its final degradation products are H₂O and CO₂), being completely suitable for drug delivery. In addition, its abundant carboxylate side chains are versatile for chemical modification for the attachment of antibodies, peptides, oligonucleotides, PEG, fluorophores, chemotherapeutic drugs, or other anti-cancer agents [41,47,50,53,54]. Each component in this nanobioconjugate plays a key role in its anticancer activity. Leucine ethyl ester is used for endosomal escape to ensure successful cytoplasmic delivery of AONs, since PMLA containing 40% leucine ethyl ester is membrane disruptive [41,55,56] through a membrane permeation mechanism distinctly described as a “carpet” model [56]. Anti-HER2/*neu* IgG3-(IL-2) functions as both a targeting molecule and as an immunostimulator. AON targeting laminin-411 α 4 and β 1 chains are designed to effectively inhibit the synthesis of laminin-411, thus, suppressing tumor angiogenesis.

The size of nanobioconjugate was estimated to be around 27 nm by Zetasizer Nano ZS-90 (Malvern Instruments, Malvern, UK). The amount of malic acid, antibody–cytokine fusion protein, mPEG₅₀₀₀, and AON was determined using the method mentioned in Materials and methods and the loading of each component was calculated by the ratio of each component compared to total malic acid. The nanobioconjugate was estimated to contain 0.25% antibody and 5% mPEG similar to the feed ratio. The total content of Morpholino was estimated to be 2.5% by reverse phase HPLC analysis of free Morpholino AON after the DTT reduction. Based on the measured amount of each component, the total molecular weight of the nanobioconjugate is calculated as 600 kDa.

3.2. Biorecognition and biological activity of nanobioconjugate

Expression of HER2/*neu*, laminin $\alpha 4$, and laminin $\beta 1$ chains in D2F2/E2 cells was confirmed by western blot analysis (Fig. 2A). The specific biorecognition of HER2/*neu* by IgG3-(IL-2) after conjugation to PMLA-fusion nanobioconjugate was examined by flow cytometry. The PMLA-fusion nanobioconjugate retained the ability to bind to HER2/*neu* on the surface of D2F2/E2 murine mammary cancer cells expressing human HER2/*neu* and SK-BR-3 human breast cancer cells (Fig. 2B). The binding level of the nanobioconjugate was similar to both IgG3-(IL-2) and anti-HER2/*neu* IgG3 alone, suggesting that IgG3-(IL-2) conjugated to PMLA retained the HER2/*neu* targeting properties of the antibody. As expected, binding to D2F2 cells that do not express human HER2/*neu* was not detected (data not shown) suggesting that the biorecognition of the nanobioconjugate is specific to HER2/*neu*.

To evaluate the biological activity of the IL-2 present in the nanobioconjugate we measured its ability to sustain the proliferation of murine CTLL-2 T-cell clonal cell line that is dependent on IL-2 for expansion. The nanobioconjugate elicits similar levels of [3 H]-thymidine incorporation compared with the anti-HER2/*neu* IgG3-(IL-2) alone. As expected the IgG3 antibody alone or buffer control did not show any activity in this assay (Fig. 3). These results indicate that the biological activity of IL-2 within the antibody–cytokine fusion protein was retained after being conjugated to the PMLA copolymer.

3.3. Cellular uptake and in vivo tumor targeting of the PMLA-fusion nanobioconjugate

The BT-474 human breast cancer cell line expresses high levels of HER2/*neu* (Fig. 2A). Specific cellular uptake of the PMLA-fusion nanobioconjugate through HER2/*neu* was visualized by confocal microscopy. A PMLA copolymer without anti-HER2/*neu* IgG3-(IL-2) showed only a weak intracellular signal after 1 h (Fig. 4A, upper panel). In contrast, the PMLA-fusion nanobioconjugate with anti-HER2/*neu* IgG3-(IL-2) was detected on the cell surface and in the cytoplasm within 2 min and the signal was the strongest after 60 min (Fig. 4A, lower panel). These results suggest that conjugation of anti-HER2/*neu* IgG3-(IL-2) to PMLA allows both the cell surface binding and the cellular uptake of the resulting nanobioconjugate. When nanobioconjugate is delivered into the cells the AONs escape from the endosomes by leucine ethyl ester as an endosomal escape unit. AONs are cleaved in the cytoplasm of cancer cells by intracellular glutathione and block the synthesis laminin-411.

In vivo imaging studies showed tumor targeting of the PMLA-fusion nanobioconjugate. Six hours after injection, only Alexa Fluor 680 labeled P/mPEG/LOEt/IgG3-(IL-2) [the PMLA-fusion nanobioconjugate minus the AON] showed strong accumulation at the tumor site in the whole body imaging analysis of treated mice (Fig. 4B, left panel). Another signal corresponding to the liver was also observed in these animals. This is expected since nanoparticles are known to accumulate in the liver [57]. Weak accumulation of P/mPEG/LOEt [the PMLA-fusion nanobioconjugate minus the AON and the anti-HER2/*neu* IgG3-(IL-2)] at the tumor site is probably a result of the non-specific enhanced permeability and retention (EPR) effect of nanoparticles [58]. Tumor targeting remained evident 24 h after treatment in excised tumors (Fig. 4B, right panel).

3.4. Treatment of syngeneic mammary tumor with PMLA-fusion nanobioconjugate with simultaneous activation of adaptive and innate immune responses

The anti-tumor activity of PMLA-fusion nanobioconjugate was initially evaluated using a syngeneic mouse model using D2F2/E2 murine mammary cancer cells that express human HER2/*neu*. Only the treatment with the PMLA-fusion nanobioconjugate, but not IgG3-(IL-2) significantly improved the survival of mice bearing mammary tumors compared to mice treated with buffer alone (Fig. 5). Additionally, the PMLA-fusion nanobioconjugate

was well tolerated. In order to explore immunoactivation as a potential mechanism that may contribute to anti-cancer activity of PMLA-fusion nanobioconjugate, serum from treated mice was collected, pooled and analyzed for the presence of murine anti-HER2/*neu* antibodies. The presence of murine anti-HER2/*neu* IgG1 in serum of treated animals is indicative of the induction of a humoral (T_H2) immune response, while the presence of murine anti-HER2/*neu* IgG2a antibodies suggests the induction of a cell-mediated (T_H1) immune response [29,59]. Both IgG1 and IgG2a levels were significantly increased in animals treated with the PMLA-fusion nanobioconjugate compared to animals treated with the antibody–cytokine fusion protein alone or buffer control animals (Fig. 6).

4. Discussion

We have previously established an efficient drug delivery system Polycefin™ using PMLA as a platform and have shown that nanobioconjugates composed of various anti-cancer agents can specifically target cancer cells and inhibit breast and brain tumor growth and angiogenesis [39,50]. PMLA-based nanobioconjugates overcome drawbacks of conventional chemotherapy by their ability to incorporate multiple drug combinations on a single platform and thus, more adequately target the tumor. We have now further modified the PMLA-based nanobioconjugate in an attempt to design a potential new therapeutic for HER2/*neu*-positive breast cancer and possibly overcome some of the hurdles of trastuzumab resistance.

Multi-functional nanobioconjugates are capable of targeting several oncogenic pathways resulting in a superior therapeutic effect compared to each drug alone. The new PMLA-based nanobioconjugate described here contains an antibody–cytokine fusion protein that serves to add two additional functions to our previous nanobioconjugates. First, tumor targeting through HER2/*neu* may also inhibit the proliferation of human cancer cells since the anti-HER2/*neu* IgG3-(IL-2) contains the variable regions of trastuzumab. Second, the antibody–cytokine fusion protein localizes IL-2 within the tumor microenvironment. This is expected to decrease the toxic side effects of high-dose systemic cytokine administration and enhance the immune response against the targeted cancer cells. In addition, the EPR effect of the nanobioconjugate may further enhance the tumor targeting. Moreover, the PMLA-fusion nanobioconjugate described here features AON against laminin-411 α 4 and β 1 chains, which are expressed by many cancer cells including BT-474, to suppress tumor angiogenesis. Therefore, the described PMLA-fusion nanobioconjugate provides a multi-pronged attack against the targeted cancer cells. It is expected that this will increase the tumor targeting ability of the nanobioconjugate resulting in enhanced anti-cancer activity with decreased potential side effects.

Synthesis of the PMLA-fusion nanobioconjugate required multiple steps and chemical reactions. It is important that the antibody–cytokine fusion protein did not lose its ability to bind the targeted antigen, HER2/*neu*, after its covalent conjugation to PMLA. Similarly it is also important that the bioactivity of IL-2 within the antibody–cytokine fusion protein was also retained within the PMLA-fusion nanobioconjugate. Our results demonstrate that the anti-HER2/*neu* IgG3-(IL-2) enhanced drug internalization into the HER2/*neu* overexpressing tumor cells. Therefore, the antibody–cytokine fusion protein retains its antigen binding and IL-2 bioactivity after covalent conjugation to PMLA. Furthermore, *in vivo* accumulation of the PMLA-fusion nanobioconjugate within the tumor area was significantly higher than that of PMLA copolymer without the antibody–cytokine fusion protein. This further confirms the successful conjugation of the fusion protein anti-HER2/*neu* IgG3-(IL-2) to PMLA, similar to our previously published data using therapeutic antibodies [37].

Laminin-411 was previously identified as a critical angiogenic marker for breast cancer [38,41,47]. It is expressed both by the tumor cells and in the tumor neovasculature. We have achieved successful suppression of laminin-411 synthesis with Morpholino AON using PMLA-based nanobioconjugates. Here we found laminin-411 expression in the murine mammary cancer cell line D2F2/E2. Therefore, it is expected that the AONs block the synthesis of tumor vascular protein expression, laminin-411 in these cells.

In an initial anti-tumor study the PMLA-fusion nanobioconjugate demonstrated significant anti-tumor activity *in vivo* and showed an immunosuppression pattern consistent with this effect. This effect was observed despite the fact that only two treatments of the nanobioconjugate were administered. The effective *in vivo* treatment possibly resulted from simultaneous: 1. Specific delivery of AONs to prevent synthesis of laminin-411; 2. Blockage of the HER2/*neu* receptor; and 3. Activation of humoral and cellular immune responses. Interestingly, treatment with the PMLA-fusion nanobioconjugate significantly increased serum levels of murine anti-HER2/*neu* IgG1 and IgG2a, suggesting an enhancement of the humoral and cellular immune responses, consistent with the anti-tumor protection in mice treated with the nanobioconjugate. Further studies are needed to confirm and improve the *in vivo* efficacy of the present nanobioconjugate and understand its *in vivo* mechanism of anti-cancer activity.

5. Conclusion

The PMLA-fusion nanobioconjugate described here represents a new generation of cancer therapeutics. The multifunctional PMLA-fusion nanobioconjugate consists of key components that facilitate its multi-pronged attack against cancer cells and their neovasculature including the induction of an anti-tumor immune response. For the first time, we have successfully incorporated an antibody–cytokine fusion protein into polymeric acid-based nanobioconjugate. Our data suggest that PMLA-based nanobioconjugates may make a significant clinical impact on the treatment of HER2/*neu*-positive malignancies.

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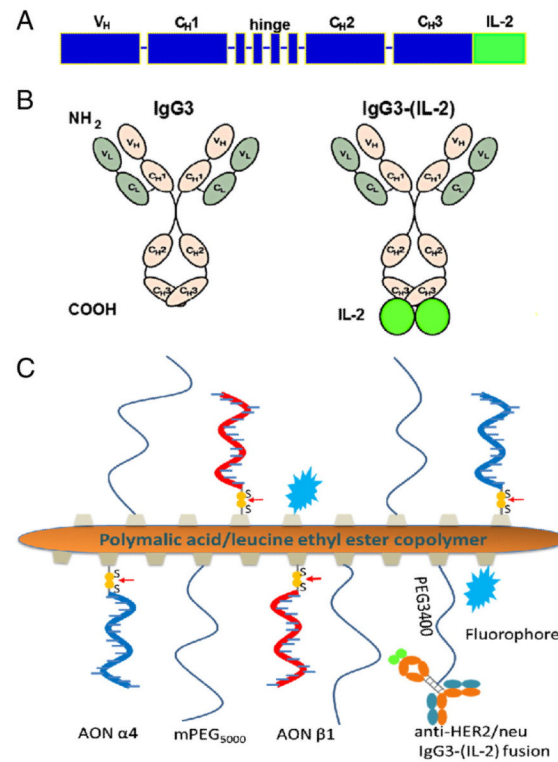


Fig. 1. Schematic representations of anti-HER2/*neu* IgG3-(IL-2) and the PMLA-fusion nanobioconjugate. A) Scheme of genes encoding the heavy chain of the anti-HER2/*neu* IgG3 fusion protein. B) Schematic representation of anti-HER2/*neu* IgG3 (IgG3) and anti-HER2/*neu* IgG3-(IL-2) antibody-cytokine fusion protein [IgG3-(IL-2)]. C) Schematic representation of PMLA-fusion nanobioconjugate [P/mPEG/LOEt/IgG3-(IL-2)/AON($\alpha 4\beta 1$)].

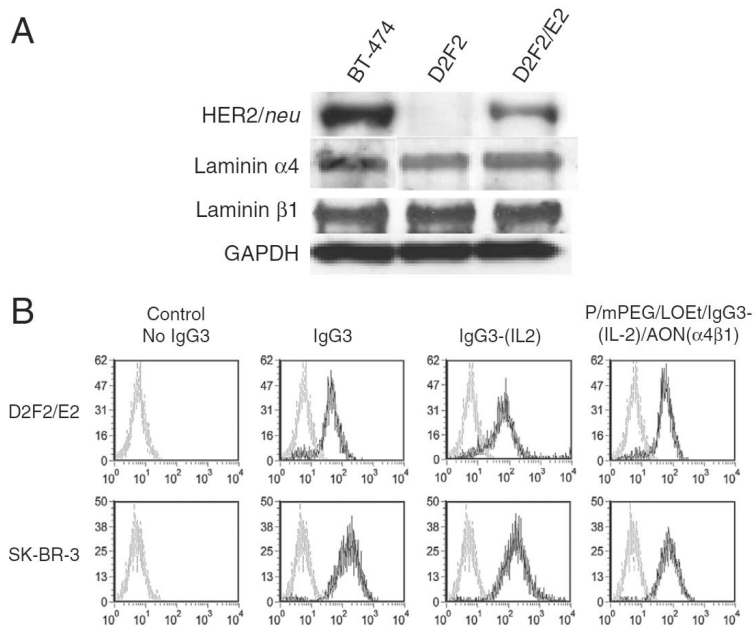


Fig. 2. Expression level of anti-HER2/*neu* and the specific binding to human anti-HER2/*neu*. **A)** The expression of anti-HER2/*neu*, laminin α 4, and β 1 chains in BT-474, D2F2, and D2F2/E2 cell lines was detected by western blot analysis. Expression of GAPDH was used as a loading control. **B)** Binding of various molecules to D2F2/E2 (top) and SK-BR-3 (bottom) as determined by flow cytometry. Cell lines were incubated in control buffer (gray line) or in the presence of anti-HER2/*neu* IgG3, anti-HER2/*neu* IgG3-(IL-2), or P/mPEG/LOEt/IgG3-(IL-2)/AON(α 4 β 1) (black line) all equivalent to 2 μ g of IgG3, followed by rabbit anti-human κ conjugated to FITC.

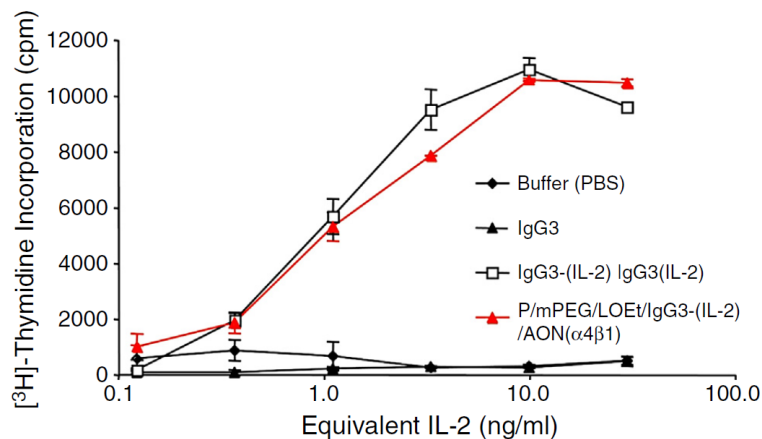


Fig. 3.

In vitro biologic activity of IL-2 within the PMLA-fusion nanobioconjugate. IL-2 activity was determined by incorporation of [³H]-thymidine into CTLL-2 cells incubated in the presence of varying concentrations of IL-2 fusion proteins and controls. Serial 1:3 dilutions of IL-2 equivalents ranging from 30 ng/ml to 0.13 ng/ml of anti-HER2/*neu* IgG3 (IgG3), anti-HER2/*neu* IgG3-(IL-2) [IgG3-(IL-2)], and PMLA-fusion nanobioconjugate P/mPEG/LOEt/IgG3-(IL-2)/AON(α4β1) were made in quadruplicate and incubated with CTLL-2 cells for 18 h followed by a 6-hour pulse of [³H]-thymidine. The error bars indicate the standard deviation.

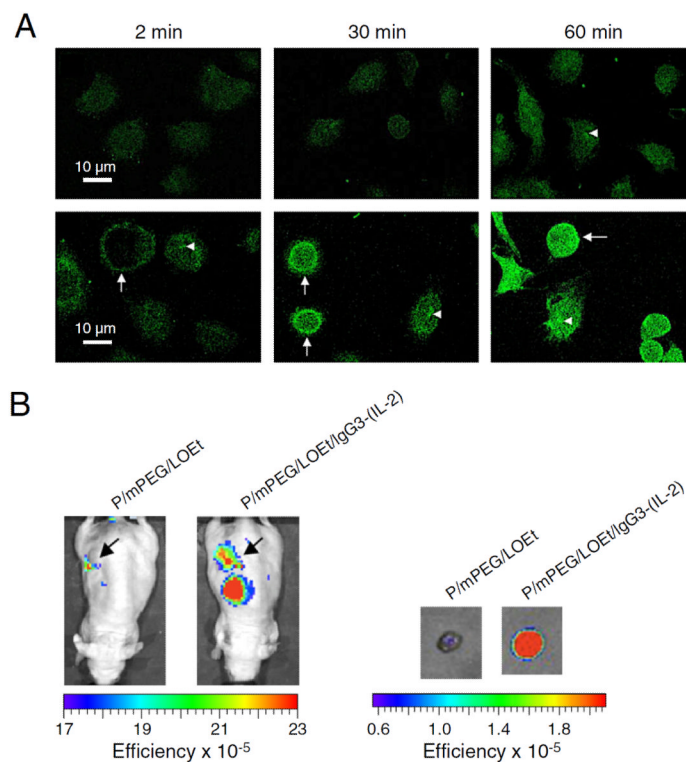


Fig. 4. Visualization of breast cancer cell targeting *in vitro* and *in vivo*. A) Internalization of PMLA-fusion nanobioconjugate in breast cancer cells visualized by confocal microscopy ($\times 40$ magnification). The human breast cancer cell line BT-474 expressing human anti-HER2/*neu* was incubated in the presence of Alexa Fluor 488-labeled PMLA copolymer P/mPEG/LOEt [without anti-HER2/*neu* IgG3-(IL-2) (upper panels)] or PMLA-fusion nanobioconjugate P/mPEG/LOEt/IgG3-(IL-2) [lower panels]. The signal was detected in cells after 2 min, 30 min, and 60 min. The location of nanobioconjugate is indicated by green fluorescence near the cell membrane (arrows), and in the endosome compartment (arrowheads). B) Whole body imaging of mice 6 h after the injection of fluorescence labeled P/mPEG/LOEt, and PMLA-fusion nanobioconjugate P/mPEG/LOEt/IgG3-(IL-2) [left panel]. Arrows mark tumor implantation site. Imaging of individual tumors extracted 24 h after treatment [right panel]. Images are representative of 4 animals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

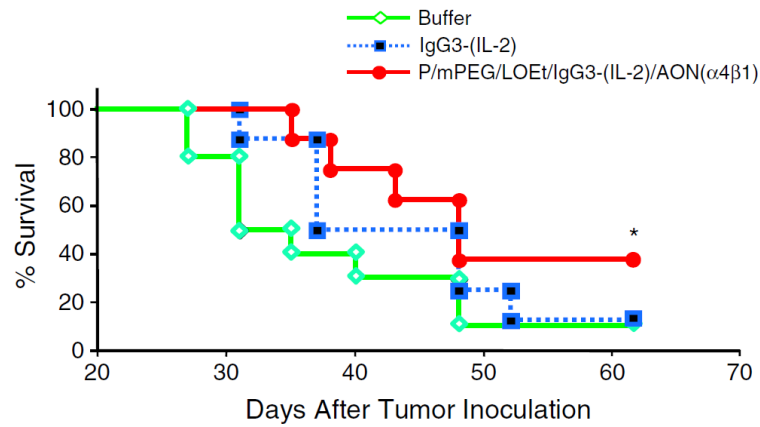


Fig. 5.

Anti-tumor activity of the PMLA-fusion nanobioconjugate in a murine syngeneic mammary tumor model. Animals bearing s.c. D2F2/E2 tumors received i.v. injections with either 10 μg of anti-HER2/*neu* IgG3-(IL-2) ($n = 8$) or the equivalent molar amount of PMLA-fusion nanobioconjugate ($n = 8$) on days 6 and 8. Control animals were injected with buffer ($n = 10$). Kaplan–Meier plot shows the survival of different treatment groups. Significant increase in animal survival is shown ($*p < 0.05$, Log-Rank test).

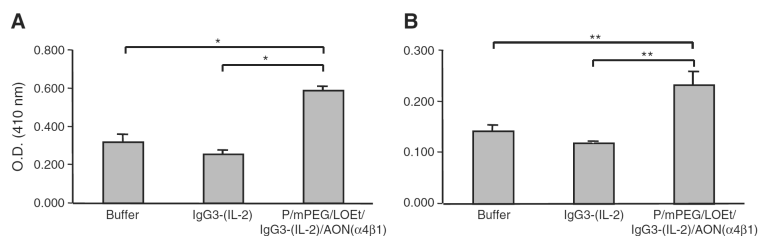


Fig. 6. The induction of cellular and humoral immune responses in animals treated with the PMLA-fusion nanobioconjugate. Blood samples were taken on day 27 after tumor inoculation from the tail vein from mice bearing D2F2/E2 tumors treated with buffer, the antibody–cytokine fusion protein, or the PMLA-fusion nanobioconjugate. Pooled samples were tested for mouse anti-HER2/*neu* IgG1 (A) as a marker of the induction of a T_H2 response and IgG2a (B) as a marker for the induction of a T_H1 response by ELISA. IgG1 level was determined using sera diluted 1:300 and IgG2a level was determined using sera diluted 1:100. Values represent the average of triplicate determinations of pooled serum aliquots and error bars indicate the standard deviation. Significant differences were determined using the Student's *t*-test (**) of $p < 0.01$ and (*) $p < 0.05$.