

Polymalic acid-based nanodrugs: Anti-tumor efficacy and host compatibility

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ABSTRACT

Polyanionic drug carriers (called nanoconjugates of the Polycefin-type) were developed that penetrate biobarriers and target molecular cancer markers of human breast and brain cancer. The carrier platform is poly(beta-L-malic acid) (PMLA), which is highly water soluble and biodegradable to CO₂ and H₂O [1]. Having a high content of reactive groups, the monomolecular carrier allows chemical attachment of multiple drug(s), targeting molecules, membrane disrupting molecules, imaging and protecting groups. We have synthesized nanodrugs highly efficient for systemic treatment in xenogeneic mouse models of primary brain cancers [2], HER2-positive breast cancer [3], triple-negative breast cancer [4], and brain metastases of breast and lung tumors. In this communication, we focus on the treatment of primary tumors. Human *in vitro* toxicity and compatibility tests were carried out for assaying a variety of physiological and immunological functions. Results of *in vivo* toxicity studies are reported for polymalic acid and for the lead drug after treatment of cancer preclinical models. Results indicated that the nanodrugs based on polymalic acid were safe and highly biocompatible under conditions of tumor treatment.

Keywords: nanodrugs, tumor treatment efficacy, polymalic acid, toxicity, immune response, brain and breast cancer treatment *in vivo*

1 MATERIALS AND METHODS

Polymalic acid carboxylic groups activated as NHS-ester were conjugated with H₂N-PEG, Leu-ethylester or Leu-Leu-Leu and 2-mercapto-1-ethylamine in a first round followed in a second round by thioether formation with Mal-PEG-Mal-S-mAb and by disulfide formation with HS-Morpholino oligonucleotides (AONs) (Fig. 1) [2-4]. Abbreviations such as P/LOEt/PEG/AON/mAb refer to nanoconjugates containing polymalic acid, leucine ethylester, mPEG, Morpholino antisense oligonucleotides, monoclonal antibodies, covalently bound to 40%, 5%, 2.5% and 0.25% of the polymer carboxyl groups. The purified nanoconjugates of Mw 300,000 to 1,000,000 were characterized chemically by content in malic acid, PEG, AON, mAb (ELISA pull-down assay), physically by hydrodynamic diameter, zeta potential, and membrane

permeation activity, biologically by effects on cell culture viability (healthy and tumor), physiologically by targeted inhibition of protein synthesis and receptor signalling (western blotting). Cell cultures were human cancer cell lines from American Type Culture Collection (Manassas, VA). *In vivo* experiments were conducted with athymic mice (CrTac: NCr-Foxn1nu Homozygous; Taconic). Human U87MG glioblastoma cells, which has been shown to express among several other tumor specific genes, the gene for laminin-411, were stereotactically implanted at 5×10^4 into the right basal ganglia of mice (n = 8 per group) [8]. For HER2-positive breast cancer, a 17 β -estradiol pellet was inserted subcutaneously 7 days before injection of 1 million BT-474 cells in 150 μ L of Matrigel into the right flanks of mice. For triple-negative breast cancer, 1 million of MDA-MB-468 cells in 150 μ L of Matrigel were injected into the right flanks of mice. When tumors reached an average volume of 120 mm³, drug conjugates were injected through the tail vein twice a week.

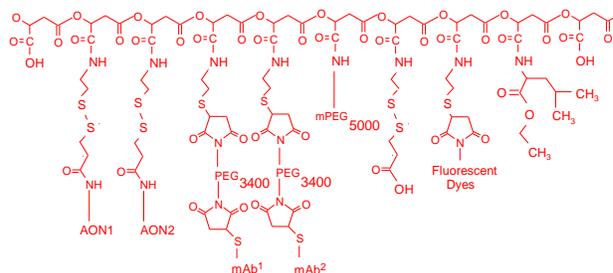


Figure 1. Schematic presentation of polymalic acid-based nanoconjugate containing PEG₅₀₀₀ (for protection), leucine ethylester (for escape from endosome), Morpholino antisense oligos (AONs), and targeting groups (mAbs).

Human *in vitro* compatibility tests for nanoconjugates (0.008-1.0 mg/mL) not containing mAb, were carried out by NCL (<http://ncl.cancer.gov>) following their specific assay protocols. *In vitro* toxicity assay for human LLC-PK1 kidney and Hep G-2 liver cells were performed. Hematological and immunological assays were platelet aggregation, erythrocyte hemolysis, plasma coagulation, and immune response: macrophage activation (NO secretion, phagocytosis), complement activation, leucocyte proliferation (immune response), and DC maturation regulating HLA-DR, HLA-ABC, CD80, CD83.

PLMA and nanopolymeric leading drug toxicity studies *in vivo* were conducted at CSMC following IV drug

administration in nude mice. Single injection at concentration 1g/kg of PMLA and 12 injections (every 3rd day) of the lead nanodrug P/LOEt/PEG/AON_{EGFR}/AON_{laminin-alpha4}/AON_{laminin-beta1}/anti-HuTfR/anti-MsTfR, each injection 37.5 mg/kg (nanodrug) containing 7.5 mg/kg (AON). In the case of PMLA toxicity, blood was analysed after 12 and 24 hrs; in the case of 12 injections, blood was analysed 14 days after the last treatment. For evaluation of CBC standard panel and blood biochemical parameters, tests included major liver, kidney and pancreatic enzymes, AST, ALT, γ -GTP, amylase, calcium, albumin, total bilirubin, alkaline phosphatase, creatinine, BUN, uric acid, cholesterol, and creatinine kinase.

2. RESULTS

2.1 Nanodrug Characterization

Lead nanoconjugates were confirmed in composition by quantitative tests for malic acid, PEG, total AON, mAbs (ELISA, protein). Presence of more than one mAb on the same conjugate was validated by pull down ELISA [6]. Specifically, dissociation constants of antigen-mAb were in the order of nM or below, the affinities for nanodrug-bound mAbs being slightly lower than for free mAbs. Purity was controlled by sec-HPLC and single species were indicated by measurement of hydrodynamic diameter (volume). These diameters fall into the range 15-35 nm, increasing with molecular weight and complexity of composition. The increase did not follow fixed increments in accordance with typical structural properties of the platform that refer to the low specific volume and the high flexibility of polymeric acid [1, 7]. Zeta-potentials were slightly negative supporting biocompatible nanodrug-cell surface interactions and are in accord with absence of toxicity.

2.2 Efficacy of Tumor Treatment

U87MG glioblastoma mouse xenografts (brain) in Fig. 2 treated 8 times with each 5 mg/kg (AON) nanodrug [2].

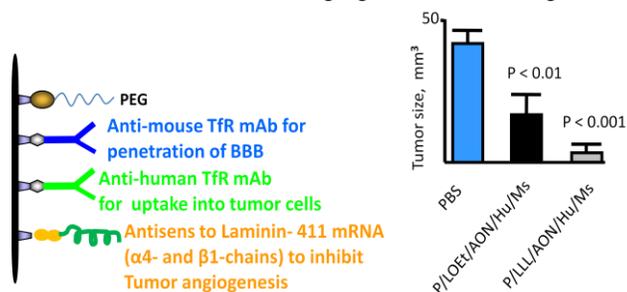


Figure 2. Treatment of human U87MG with the nanodrug shown schematically **on the left**. The black bar refers to PMLA covalently bound to either leucine ethylester (LOEt) or trileucine (LLL), at 40% of the polymer pendant carboxylates. Tumor growth inhibition **on the right** was more efficient for trileucine than for leucine ethylester.

In this nanoconjugate, the growth inhibiting drugs were antisense oligonucleotides that inhibited the synthesis of alpha-4 and beta-1 subunits of tumor expressed laminin-411 which was required for tumor specific angiogenesis [8].

HER2-positive breast cancer mouse xenograft in Fig. 3 during 8 treatments with the lead nanodrug containing AON_{HER2} (2.5 mg/kg) and each antibody (4.5 mg/kg) [3] on the **left** and with various other constructs for comparison listed on the **right**.

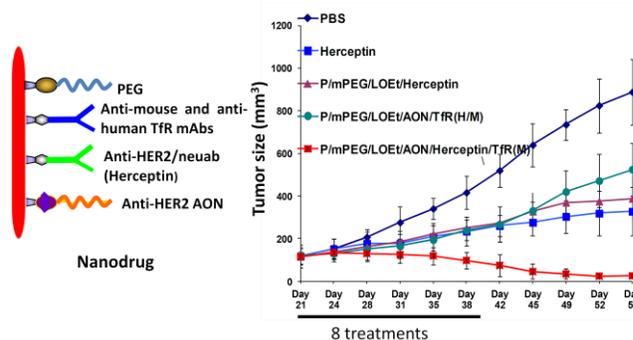


Figure 3. Treatment of human HER2-positive breast cancer xenograft with the lead nanodrug shown schematically on the **left**, where red bar refers to PMLA covalently bound to leucine ethylester at 40% pendant carboxyl groups. Tumor size vs. treatment (**right**) shown for the lead nanodrug (red), Herceptin only (blue), the lead without AON and anti-TfR mAbs (magenta), and the lead without Herceptin (green). The tumor size decreased by more than 95% after the treatment with the lead compound which contained both Herceptin and the anti-HER2-oligonucleotide (AON_{HER2}) for inactivating signaling and resynthesis of HER2.

Triple-negative breast cancer mouse xenograft was treated with the lead nanodrug containing AON_{EGFR} (2.5 mg/kg) and mAbs [4] on the **left** and with various other constructs for comparison listed on the **right** in Fig. 4.

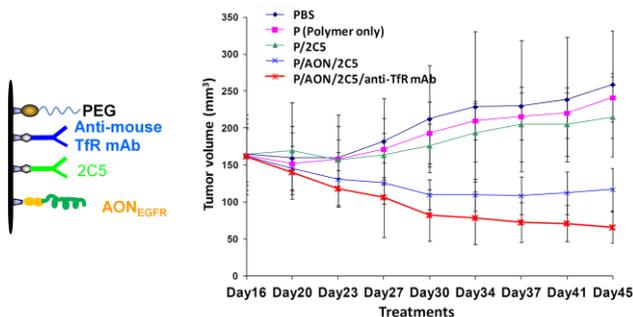


Figure 4. Treatment of human triple-negative breast cancer xenograft with the lead nanodrug on the **left**. The targeting antibody 2C5 binds to antigens typically on multiple cancer cells [9]. Tumor size dependence on number of treatments is shown on the **right** with the lead nanodrug (orange), the

<i>In vitro</i> assays for toxicity study	PMLA	PMLA/ mPEG ₅₀₀₀ (5%)/ LOEt(40%)	PMLA/ mPEG ₅₀₀₀ (5%)/ 2-MEA/ LOEt(40%)/ AON _{α-4} (0.13%)/ AON _{β-1} (0.13%)	PMLA/ mPEG ₅₀₀₀ (5%)/ 2-MEA/ LOEt(40%)/ AON _{EGFR} (0.5%)/ mAb(0.22%)
HEPG2 cells (human hepatocarcinoma), Cytotoxicity assay	None	None	None	N.d.
LLC-PK1 (porcine renal proximal tubule) Cytotoxicity assay	None	<0.06 mg/mL	<0.08 mg/mL	N.d.
Hemolysis	None	None	None	None
Platelet aggregation	None	None	None	None
Platelet aggregation, collagen induced	Inhibition	Inhibition	Inhibition	Activation
Interference with prothrombin time (PT)	None	None	None	None
Interference with thrombin time	Increased	None	None	None
Proliferation of human leukocytes	None	<0.4 mg/mL	<0.4 mg/mL	N.d.
Nitric oxide secretion by macrophages	None	None	None	N.d.
Chemotaxis	None	None	None	N.d.
Phagocytosis, effect on phagocytosis of Zyosan A	None	None	None	N.d.

Table 1: Results for human *in vitro* compatibility of tested compounds in a concentration range with maximal 1 mg/mL.

lead without anti-MsTfR mAb (blue), the lead without AON and anti-MsTfR (green) and polymer only (magenta). The leading drug demonstrates the best treatment result in tumor size reduction.

2.3 Biocompatibility

Human in vitro compatibility tests were carried out for PMLA and the nanoconjugate precursors of the nanodrug to treat human glioma U87MG, and for the lead nanodrug containing AON_{EGFR} and IgG2a-κ (Table 1). The toxicity results were the same for cell viability and membrane integrity tests. In previous investigations, we could refer the toxicity to apoptosis induced by the leucine ethylester (LOEt) endosome escape unit, which bound pH-independently to membranes and induced their lysis at concentrations >0.1 mg/mL [2]. The alternatively used endosome escape unit, trileucine (LLL), was active only at pH < 6.0 and was nontoxic [2, 10]. The compounds did not induce significant aggregation of human platelets. The inhibition of collagen-induced aggregation was likely the result of pendant carboxylates on the polymers. The compounds did not interfere with the plasma coagulation as measured by the prothrombin and thrombin times. The observed inhibition by times was due to special effects of the compounds. Since precursors showed inhibition of collagen induced platelet coagulation, while the lead compound was an activator, it is difficult to conclude whether the lead displays the same anticoagulant properties of the precursors. Border line complement activation far below the level of FDA-approved Doxil was assayed by

testing iC3b, and Bb. Also, non-human systems (Mini Pig, Guinea Pig, rat, mouse) did not show an activation. Furthermore, The tested compounds did not induce proliferation of human leukocytes indicating that polymeric acid-based nanoconjugates did not immunostimulate unprimed human leukocytes. The nanodrugs tested were not taken up by macrophages nor were they found to induce chemotaxis and oxidative burst. Measuring numbers of cells expressing HLA-DR, HLA-ABC, CD-86, CD-83, CD-80, CD-14 after exposure of Monocyte Derived Dendritic cells to 0.008 - 0.2 mg/mL PMLA indicated that maturation of the dendritic cells was not induced. In addition, no effect on LPS induced maturation was observed. After extraction with Triton X-140 [11], endotoxin resulted in lower than FDA-set standard levels for toxicity as measured by LAL-endpoint assay and pyrogenic rabbit test. NCL confirmed that PEG was not released from the compounds in solution and during lyophilization.

2.4 In vivo Toxicity Studies

In vivo biocompatibility of PMLA at 0.1 g/kg and 1.0 g/kg, injected into nude mice via the tail vein, was assayed in the blood after 12 and 24 hrs. Similarly, after 12 injections, each 37.5 mg/mL of the lead drug against triple-negative breast cancer (see Materials and Methods), no toxicity was noticed by comparing CBC standard panel and blood biochemical parameters for PBS and nanodrug-injected nude mice.

3. CONCLUSION

The presented data show results for effective treatment of primary brain and breast tumors. The results of biocompatibility studies indicate that the tested nanodrugs and precursor nanoconjugates on polymalic acid basis, and polymalic acid itself, are excellently tolerated by human kidney and liver cells *in vitro* without hematological changes and immune toxicity, and by nude mice *in vivo*. Together, the proven preclinical treatment efficacy of multiple tumor models *in vivo*, the biodegradability and the host compatibility are reasons to position nanoconjugates of the Polycefin family at the top of the list of nanodrugs suitable for cancer treatment.

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