Nanoconjugates of Poly(malic acid) with Functional Modules for Drug Delivery

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ABSTRACT

Nanoconjugates of β-poly(L-malic acid) (PMLA) have been synthesized with modules active in drug delivery, drug release, blocking of tumor-affiliated mRNA by antisense oligonucleotides, penetration of blood and cellular barriers, tissue targeting, membrane permeation, fluorescence, and protection. PMLA of microbial origin is non-toxic, non-immunogenic, biodegradable and highly suited as a scaffold for tailored nanoconjugate chemistry. The nanoconjugate of 550 kDa allowed fluorescence imaging of brain tumor and breast cancer implanted on mouse, targeted on the basis of tumor tissue-inherent enhanced permeability and retention (EPR) and antibody recognition. The nanoconjugates were designed to inhibit tumor growth by preventing angiogenesis.

Keywords: antisense oligonucleotides, antibodies, tissue targeting, fluorescence imaging, tumor angiogenesis

1 INTRODUCTION

Nanoconjugates have been synthesized from biogenic and synthetic polymeric scaffolds by covalent bonding of pendant functional groups with small drug molecules, proteins (mAbs), oligonucleotides and/or membrane disrupting moieties. Ideally, the conjugates should be soluble, non-toxic, non-immunogenic, and biodegradable. To maximize tumor treatment efficiency, they should contain tissue targeting, cell invading, and tumor inhibiting drugs. A consortium of prodrugs should be delivered and activated in targeted cells to synergistically unfold a highly efficient inhibition of tumor cell growth and metastasis. While a few of these demands are found fullfilled in existing drug delivery systems, an optimization that includes many, possibly all of these criteria, has not been reported. Drug development involves two stages: the physical targeting and the antitumoric action of the delivery system. The target protein, laminin-8 (α4β1γ1), is a vascular basement membrane (BM) component that was overexpressed in high-grade gliomas, invasive breast cancer, and its metastasis compared to normal brain and breast [1,2]. Expression of laminin-8 correlated with shorter GBM recurrence time and poor patient survival [2,3]. Laminins are major BM components mediating cell adhesion, migration, and angiogenesis.Targeting may be followed by animal imaging when the delivery system (optionally) has been attached to a fluorescent reporter dye. We have succeeded in synthesizing a drug delivery system conform with the above mentioned criteria. We report here the drug properties studied by *in vivo* imaging, and the targeting of laminin-8, important for tumor microvessels development.

2 MATERIALS AND METHODS

PMLA of M_w = 50,000 (polydispersity M_w / M_n = 1.3) was highly purified from cultures (plasmodia) of the slime mold *Physarum polycephalum*. Morpholino™- 3′-NH_2 antisense oligonucleotides AGC-TCA-AAG-CCA-TTT-CTC-CGC-TGA-C to laminins α4 (MORPH-AON-1) and CTA-GCA-ACT-GGA-GAA-GCC-CCA-TGC-C to β1 (MORPH-AON-2) (constituents of laminin-8) [1,2] were custom made by Gene Tools (Philomath, OR). Mouse anti-rat mAb (clone OX-26, IgG_{2a},κ, 1 mg/mL in PBS; cross-reacting with human as shown in this work) and rat anti-mouse mAb (clone R17217, IgG_{2a},κ, 1 mg/mL in PBS, cross-reacting with human, results not published) to transferrin receptor (CD71) were obtained from Chemicon International (Temecula, CA).

N-Hydroxy-succinimide-activated carboxyls of PMLA were substituted by H_2N-PEG, L-leucine (forming membrane disrupting units), and 2-mercaptoethylamine. mAb(s) (e.g. transferring receptor antibody), disulfide-interspaced H_2N-morpholino antisense oligonucleotdes to laminin-α4 and laminin β1, and fluorescent dyes (optionally) were coupled to pendant sulfhydryls. Nanoconjugates and intermediates were purified by molecular sieving and verified by HPLC, SDS-PAGE, thinlayer chromatography, chemical tests. For detailed description see [3]. Nude mice [Tac:Cr:(MCr)-$Foxn^{nu}$] received intracranial injection of 5 x 10^4 U87MG human glioma cells. Or nude mice after the implantation of the 17β-estradiol pellet received 5x10^7 BT-474 human breast cancer cells (EGFR-positive) injected subcutaneously into the left posterior mid-dorsum. After manifestation of the

tumor, 100 µl of drug solution was intravenously injected via the tail vein at a concentration of 2.5 mg/kg [4]. The drug distribution was followed by fluorescence imaging at different time points. These experiments were done with a customized macro-illumination and detection system for live animals (MISTI System [5]; see also [3]).

3 RESULTS AND DISCUSSION

3.1 Nanoconjugate Synthesis

The drug delivery device was synthesiszed in a fashion allowing high yields, full consumption of reagents and efficient purification, starting with (N-bromosuccinimide ester, NHS)-activation of pendant –COOH under exclusion of water, then amide coupling of various groups via their amino group, and coupling via the thus introduced sulfhydryl functions. The schematic structure of the nanoconjugate is shown in Fig. 1. Appropriate variants of this master conjugate were synthesized to prove its chemistry and functions as a drug delivery system.

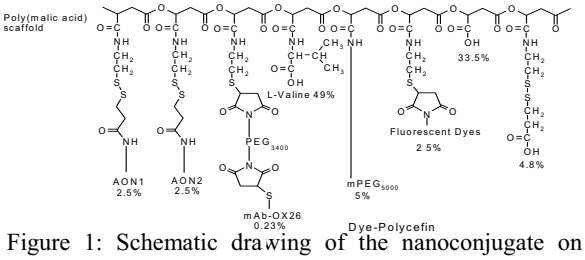

Figure 1: Schematic drawing of the nanoconjugate on poly(malic acid)-basis. Per cent values refer to total malate content (100%). AON1, AON2, morpholino antisense oligo nucleotides to laminin α4 and laminin β1 (constitutents of laminin-8). Dyes were either Fluorescein or AlexaFluor 680.

3.2 Nanoconjugate Physical and Chemical Properties

The nanoconjugate was highly water soluble and pure by size exclusion HPLC, thin layer chromatography, testing for functional groups like amino acids, sulfhydryls, of the nanoconjugate, and devoid of small molecular impurities. Stoichiometries of conjugated ligands (composition) were controlled at the levels of each of the intermediates, either by achieving reaction completions or by appropriate cleavage into fragments followed by their chemical quantitation. The average molecular mass according to the molecular composition in Fig.1 is 550 kDa. mAbs are conjugated to the PMLA scaffold at H-chains. Membranes are destabilized and disrupted by leucine containing stretches of the scaffold. Under conditions found in the cytoplasm (3 mM GSH), the drug (morpholino antisense oligonucleotides) is cleaved from the PMLA scaffold [3].

3.3 *In vitro* Properties

The nanoconjugate did not change cell viability, measured for cultures of two human gliomas, U87MG and M059K, and normal human astrocytes, HAST 040 [3]. The numbers of treated cells were compared with those of replicate cultures without treatment (taken as 100%). Cell viability for each cell line in two separate experiments was higher than 90%. Untreated controls and treated cultures did not differ significantly from each other ($p>0.05$). Based on these data we concluded that the nanoconjugate at concentration 1.4 and 14 µM did not exert toxic effects on these cell lines.

In the *in vitro* studies with human U87MG and T98G glioblastoma cell lines it was shown that the nanoconjugate was internalized into U87MG glioma cultures.

Target Inhibition. To show that the nanoconjugate escaped its primary cellular location through membrane disruption into the cytoplasm, the inhibition of laminin-8 chain synthesis/ secretion *in vitro* was studied. The nanoconjugate efficiently blocked secretion of laminin α4 and β1 chains in the culture medium of glioma cells as shown by semi-quantitative Western blot analysis of conditioned culture media sampled after 6 days of culture (secreted human fibronectin served as gel loading control) as shown in Fig. **2**. In cultures treated with nanoconjugate the expression of both chains was either significantly reduced (α4) or completely abolished (β1).

Mechanism After treatment with Fluorescein-labeled nanoconjugate (Fig. 1), fluorescence was localized near the cell membrane and in early endosomes after 10 min and inside the cells after 20 min after addition of the drug (Fig. 3B). When U87MG were co-stained with fluorescent endosomal marker FM 4-64 (visualized with rhodamine filter), the staining for both Fluorescein-nanoconjugate and FM 4-64 was co-localized after 30 min (Fig. 3C), indicated by the yellow color after superposition of red and green. Internalization was mediated by binding of mAbOX-26 residue of the nanoconjugate to human transferin receptor at the cell surface as indicated by the crossreaction of mAb (rat to human) as shown in Fig. 3A. Also, when cells were pre-treated for 10 min with mAb OX-26 in order to block the cell surface transferrin receptor, and then incubated with nanoconjugate, the drug was not internalized, and fluorescence was not seen inside the cells confirming that conjugate delivery occurred by the mechanism of transferrin receptor-mediated endocytosis. Fluorescein-labeled nanoconjugate(-mAb) administered at the same concentration also passed through the cell membrane, however at much lower rate in accordance with the known EPR-effect for macromolecules [6] (results not shown).

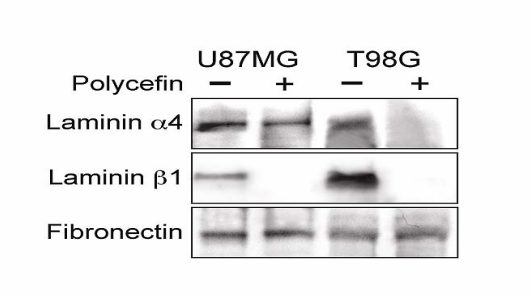

Figure **2**: *In vitro* targeting and inhibition of laminin chain synthesis by treatment with nanoconjugate. Western blots of conditioned media of both glioma cell lines, U87MG and T98G, containing α4 and β1 chains of laminin-8. Secretion of both laminin chains was markedly decreased.

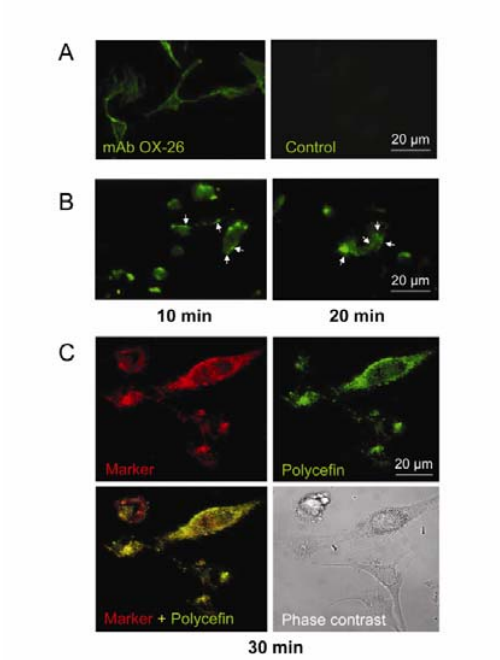

Figure **3**: Nanoconjugate delivery into cultured human glioma U87MG cells. Confocal microscopy. A, rat mAb Ox-26 cross reacted with the surface of human cells. B-C, Nanoconjugate (green) uptake by endosomes (red).

3.4 *In vivo* studies. Nanoconjugate tumor accumulation

The *in vitro* experiments have demonstrated the endosomal uptake and inhibitory antisense function of the oligonucleotides. The following *in vivo* experiments were carried out with a leucine derivate (in place of valine in Fig.1) conjugated to AlexaFluor 680. Fig. 4 shows fluorescence imaging of human glioma implanted in nude mice brains. A similar result was obtained for implanted human breast cancer. Fig. 5 shows fluorescence imaging in this case after i.v. injection of the nanoconjugate in the absence of bound mAb to show the EPR-effect.

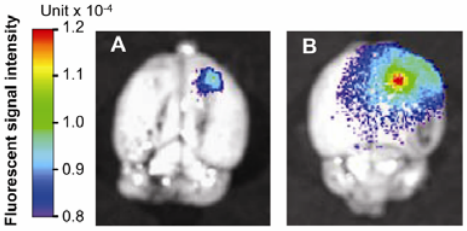

Figure 4: Xenogen IVIS 200 imaging of fluorescent nanoconjugate in brain glioma, (A) nanoconjugate with, and (B) nanoconjugate without mAb to transferrin receptor. Without mAb, fluorescence accumulated in brain tumor 72 hours after i.v. injection in agreement with EPR-effect. AlexaFluor 680 alone was negative. However, complete nanoconjugate with the targeting antibody showed significantly higher tumor retention. Imaging was carried out after flushing perfused brains with sodium buffered saline.

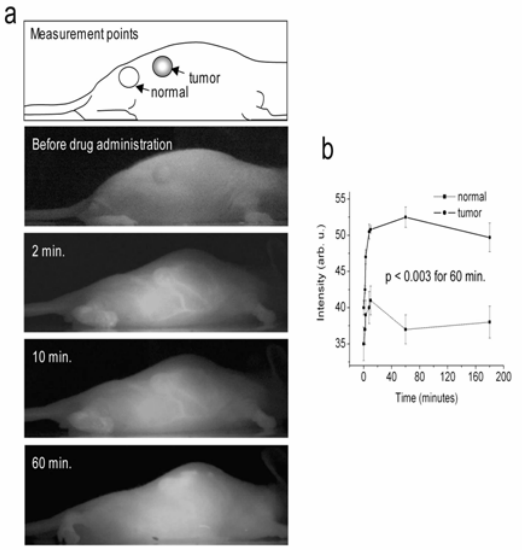

Figure 5: *In vivo* fluorescence images (MISTI imaging system) of mice with human breast tumor (EGFR-positive BT-474 line) before treatment and in 2, 10, and 60 min. after intravenous injection of AlexaFluor 680-labeled nanoconjugate not conjugated with mAb to transferring receptor. Fluorescence intensity analysis was performed by selecting small areas within the tumor and normal regions as shown in a cartoon (a; top). The time course fluorescence intensities in tumor area (solid line) and normal area (dotted line) are shown in graph (b). Nanoconjugate circulated in blood vessels in early phase (2-10 min), but declined after 60 min (a, b). However, the drug kept retaining in tumor tissues even after 60 min (b).

3.5 Conclusion

A new prototype of nanoconjugate, was synthesized and shown to target delivery of antisense oligonucleotides and monoclonal antibodies to brain and breast cancer.

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