Nanobioconjugates for Triple-Negative Breast Cancer Treatment.

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ABSTRACT SUMMARY
Covalent nanobioconjugates have been synthesized based on poly malic acid (PMLA), targeting triple-negative breast cancer (TNBC). They significantly inhibited tumor growth in mice by blocking synthesis of laminin-411 a tumor vascular wall protein and angiogenesis marker, and epidermal growth factor receptor (EGFR). Nanobioconjugates toxicity and biocompatibility were evaluated in vitro and in vivo. The present study demonstrates that the dual-action nanoconjugate is highly effective in preclinical TNBC treatment without side effects, supported by hematologic assays data.

INTRODUCTION
The use of polymeric based multitargeted drug delivery systems (DDS) in cancer therapy is increasing in popularity because they are less immunogenic than protein-based vectors, and allow repetitive administration without acute or chronic host immune response.¹

EXPERIMENTAL METHODS
Highly purified, endotoxin-free poly(b-L-malic acid), Mw (weight-averaged) 70 kDa, polydispersity 1.1, was prepared from the culture broth of Physarum polycephalum and purified by DEAE cellulose, ethanol precipitation, size exclusion chromatography and lyophilization.

Nanoconjugates synthesis involves chemical activation of PMLA pendant carboxyl groups, substitution by amide formation yielding the preconjugate containing the endosome escape unit leucine ethyl ester and PEG. To complete the nanodrug synthesis, activated antibodies and activated AONs were attached. Antibodies were activated by disulfide reduction and coupling to MAL-PEG3400-MAL that was conjugated at the maleimidyl group with preconjugate-SH. AONs were activated by reacting with N-succinimidyl 3-(2-pyridyldithio)propionate.

The product, formed disulfide bonds with sulphydryl groups of the antibody-S-preconjugate yielding the nanoconjugate. The composition of the final nanoconjugate was validated by chemical group analysis of malic acid, AONs, mAbs, and mPEG5000.

Athymic female mice [Tac:Cr:(MCR)-Foxnmm] were inoculated into the right flanks with $1 \times 10^7$ EGFR-positive breast cancer MDAMB-468 cells.² The treatments started when tumor sizes reached an average of >120mm³. Mice were equally distributed into four treatment groups and injected with sterile PBS or P/AON⁰EGFR MsTfR-HuTfR (12.5 mg/kg by AON) or P/AON⁰α4,β1 MsTfR-HuTfR (25 mg/kg by AON) or P/AON⁰EGFR,α4,β1-MsTfR-HuTfR (37.5 mg/kg by AON) into the tail vein twice a week. Two independent animal studies were conducted with 6 and 12 treatments.

Figure 1. Composition of PMLA-based nanobioconjugate for TNBC treatment. AONs to chains α4 and β1 of laminin-411 and EGFR, carboxylates for water solubility, human and mouse transferrin receptor antibodies, mPEG for solubility and protection, capped sulphydryl and endosome escape unit.
RESULTS AND DISCUSSION
The nanoconjugates inhibit growth of EGFR-positive triple-negative breast tumors in nude mice. We investigated the therapeutic effect of the novel nanobioconjugates by i.v. treatment using the subcutaneous inoculation of human MDA-MB-468 cells overexpressing EGFR. The in vivo experiments included PBS (control), the complete dual action nanodrug P/AON\textsubscript{EGFR,\alpha\textsubscript{4},\beta\textsubscript{1}}-MsTfR-HuTfR, and the single-action nanoconjugates P/AON\textsubscript{EGFR-MsTfR-HuTfR} and P/AON\textsubscript{\alpha\textsubscript{4},\beta\textsubscript{1}}-MsTfR-HuTfR. Tumor growth inhibition data are based on two experiments with 6 and 12 treatments.

![Figure 2](image)

Figure 2. Treatment of nude mice bearing TNBC. (a) Efficacy showing reduction in tumor size during 6 and 12 treatments. Highest efficacy was obtained with complete dual-action drug when all three AONs were together on the same platform. (b) Western blots of tumor protein extracts upon various treatments showing effects on the expression of EGFR, laminin \(\alpha\textsubscript{4}\) chain, laminin \(\beta\textsubscript{1}\) chain and pAkt. The strongest inhibitory effect is obtained with dual-action nanoconjugate P/AON\textsubscript{EGFR,\alpha\textsubscript{4},\beta\textsubscript{1}}-MsTfR-HuTfR. Results are shown for three mice after 12 treatments.

The data suggest that the complete dual-action drug showed synergy with the anti-EGFR single action drug (Fig.2a). To test at the molecular level for possible synergy between these AON-based nanodrugs, tumors of three mice per group were extracted after the end of 12 treatment-experiment and proteins examined by western blotting (Fig.2b). P/AON\textsubscript{EGFR-MsTfR-HuTfR} reduced the amount of both EGFR and its downstream signaling intermediate pAkt, but had no effect on the expression of laminin \(\alpha\textsubscript{4}\) and \(\beta\textsubscript{1}\). Similarly, treatment with P/AON\textsubscript{\alpha\textsubscript{4},\beta\textsubscript{1}}-MsTfR-HuTfR had no effect on EGFR, but reduced the amounts of laminin \(\alpha\textsubscript{4}\) chain and pAkt. In contrast, treatment with dual-action nanodrug P/AON\textsubscript{EGFR,\alpha\textsubscript{4},\beta\textsubscript{1}}-MsTfR-HuTfR significantly inhibited the expression of EGFR, pAkt and laminin \(\beta\textsubscript{1}\) and \(\alpha\textsubscript{4}\) chains, consistent with cross-talk between EGFR and the laminins through laminin-binding integrin receptors.\textsuperscript{3}

During 12 treatments, i.e. i.v. injections every 3 days of nanoconjugates, nude mice did not exhibit adverse physical effects such as body weight loss, morbidity, or death indicating that all treatments were well tolerated. Gross-and micro-histopathology of all major organs/tissues by H&E staining did not reveal any visible morphologic changes. Complete blood count cell counts, metabolic and chemistry panels demonstrate normal parameters in all blood tests for all the injected nanoconjugates compared with untreated group of mice and the control group.

CONCLUSION
The present study demonstrates that multiple targeted nanoconjugates based on nontoxic, non-immunogenic polymeric acid are highly effective in preclinical Triple-Negative breast cancer treatment without side effects, supported by hematologic data.

REFERENCES

ACKNOWLEDGMENTS
The work was supported by NIH grants R01 CA123495, U01 CA151815 and R01 CA136841 and a grant from Arrogate, Inc., Tarzana, CA, USA.