

審査の結果の要旨

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Sialic acid (SA) is the the terminal group of the cell surface glycan chain, which regulates a range of pathological processes. SA is involved in various cellular activities in healthy tissues. Altered glycosylation is a trademark of almost all type of cancers regardless of the origin and stage. SA has been reported to be involved in immune modulation and support of cancer cells in immune evasion to create an immunosuppressive microenvironment. Hypersialylation of cancer cells also promotes migration and apoptosis resistance, which are beneficial for tumor growth and correlated with aggressiveness and poor prognosis for cancer patients. Thus, SA represents a broad marker for tumor targeting of therapeutic agents. Nevertheless, SA is also expressed in healthy tissues, thus, hindering the development of such targeted strategies. SA can be recognized by lectins and antibodies though they are yet to be used systematically due to their immunogenicity and lack of selectivity to tumor SA. Thus, the development of ligands capable of recognizing tumor SA, while avoiding interaction with SA in healthy tissues, is still necessary for advancing SA targeted strategies. Boronic acids can form reversible covalent interactions with diol-containing molecules. While the binding of boron to diol-containing molecules is commonly promoted at basic pH, the binding constant between these agents drops at neutral and acidic pHs. However, the complexes between phenylboronic acid (PBA) and SA are stable at pH lower than pKa. This selectivity of PBA for SA at acidic pH can be useful for designing ligands specific to SA on tumor cells, as intratumoral pH is lower than the pH of healthy tissues, pH 6.5 and pH 7.4, respectively. Thus, PBA have been reported to selectively recognize SA overexpressed on cancer cells to improve the tumor targeting ability of polymeric micelles. Besides cancer cells, researchers have also explored the idea of using PBA as a synthetic lectin to activate T cells, inducing lymphocyte proliferation, which could be applied as an IL-2 receptor stimulant. As the interaction between SA and PBA is low, in this dissertation we have focused on finding PBA derivatives with higher binding affinity to SA at intratumoral pH, which can further improve the tumor targeting ability.

In **Chapter 1**, general introduction of background knowledge was presented. A brief outline of the impact and implications of nanomedicine especially polymeric micelles in cancer therapy was introduced. The approaches and benefits of ligand-based nanomedicine to specifically target cancer cells was summarized. Different targets and characteristics unique to cancer cells were introduced for the better understanding of the ligand-based design strategies. The advantage and features of SA as a target molecule for anti-cancer therapy was discussed including their overexpression in resistance cancer stem cells (CSCs). Finally, summary of various treatment strategies directed towards SA in tumor and advantages of PBA as a targeting molecule for SA was outlined.

In **Chapter 2**, the prospect of novel 5-boronopicolinic acid (5-BPA) to use as a ligand on nanoparticle for targeted cancer therapy was evaluated. Overexpression of SA in different *in vitro* cultured murine and human cancer cell lines were verified. Relatively higher expression of SA in tumor samples compared to normal tissues collected from head and neck cancer patients was also confirmed. The concept of pH-dependent binding of 5-BPA molecule to SA on the cancer cell surface was proved *in vitro*. Firstly, the improved ability of 5-BPA to block SA specific lectin binding at pH 6.5 was demonstrated. Then, additional experiment was performed by using rhodamine conjugated 5-BPA and directly observing higher binding of 5-BPA-rhodamine to cell surface SA, which was diminished upon removal of SA through sialidase treatment. Retention of binding constant of 5-BPA after polymer conjugation was confirmed through ARS (Alizarin Red S) assay using 5-BPA-PEG-acetal as model polymer. Finally, the superiority of 5-BPA over PBA as a ligand molecule for targeting cancer cells was validated using fluorescent dye-labeled 5-BPA-8-arm-PEG and PBA-8-arm-PEG. 8-arm-PEG was chosen due to hexaglycerol shape and multiple conjugation site for boronic acids to imitate similar multivalency properties demonstrated by the polymeric micelles. 5-BPA-8-arm-PEG demonstrated improved cellular uptake at pH 6.5 and higher tumor accumulation compared to PBA-8-arm-PEG probably by enhanced interaction with intratumor SA in acidic tumor microenvironment.

In **Chapter 3**, preparation and characterization of polymers and micelles installed with PBA derivatives are reported. To prepare polymeric micelles, poly(ethylene glycol)-*b*-poly(L-glutamic acid) (PEG-*b*-PLGA) block copolymers with narrow weight distribution and 40 units of PLGA side chain were synthesized and characterized. PBA and 5-BPA were conjugated to the end-terminus of acetal-PEG-*b*-PLGA and azide-PEG-*b*-PLGA respectively. Alexa 555- and Alexa 647-conjugated methoxy-PEG_{10K}-*b*-PLGA were prepared to construct fluorescent micelles. Moreover, DACHPt-loaded polymeric micelles (DACHPt/m) were constructed with 50% surface ligand (5-BPA or PBA). All the micelles demonstrated similar size, drug loading and surface charge, thus allowing us to compare their biological activity for the ligand efficiency.

In **Chapter 4**, the biological activities of 5-BPA-installed DACHPt-loaded micelles (5-BPA-DACHPt/m) were assessed in the CSC-rich head and neck cancer cell line HSC2, and compared with non-ligand DACHPt/m and PBA-installed micelles (PBA-DACHPt/m). Quantification of internalized Pt revealed higher cellular uptake of 5-BPA-DACHPt/m compared to other micelles at pH 6.5. The improved cellular uptake of ligand-installed micelles at pH 6.5 was reduced upon cleaving of SA. Relatively lower IC₅₀ value also demonstrated by 5-BPA-DACHPt/m compared to DACHPt/m confirmed the effectiveness of ligand-mediated targeting. Moreover, cellular studies also revealed the ability of 5-BPA-DACHPt/m to reduce SA rich CSC population. *In vivo*, 5-BPA-DACHPt/m showed significantly higher tumor accumulation and retention 48 h after intravenous injection compared to other micelles in subcutaneous HSC2 tumor model. Finally, antitumor study against orthotopic head and neck HSC2 tumor model revealed the extraordinary ability of 5-BPA-DACHPt/m to suppress tumor growth and improve survival compared to both DACHPt/m and PBA-DACHPt/m treated groups. Assessment of CSC population in the tumors after treatment revealed significantly lower fraction of CSCs in 5-BPA-DACHPt/m treated animals.

In **Chapter 5**, we evaluated potential of PBA-conjugated polymers as synthetic lectins for immunostimulation. The ability of 8-arm-PEG-PBA and 8-arm-PEG-5-BPA to induce T cell proliferation was validated using immortalized human T lymphocytes (Jurkat cells) at pH 7.4 and pH 6.5. The activation ability demonstrated by the polymers were not replicated by small molecule PBA or 5-BPA. T cells collected from mouse lymph nodes also demonstrated 3-fold higher proliferation upon incubation with 8-arm-PEG-PBA, but not with free PBA. Treatment with 8-arm-PEG-PBA also showed significantly higher induction of CD4⁺ in cells collected from both lymph nodes and thymus. On the contrary, thymus cells that were treated with 8-arm-PEG-5-BPA demonstrated small, but significant increase on CD8⁺ cells.

In **Chapter 6**, the result and significance of each chapter was summarized, and the future perspective for 5-BPA-installed antitumor strategies were described.

In summary, we have developed 5-BPA as a potent ligand for tumor targeting, as validated through the modification of small molecules, polymers and polymeric micelles loading anticancer drug. Moreover, we demonstrated through extensive cellular and animal studies that 5-BPA-DACHPt/m improved tumor targeting and antitumor efficacy due to specific interaction of 5-BPA with SA at intratumoral acidic pH. Our smart ligand system not only improved delivery of cytotoxic drugs, but also were effective in eliminating resistant CSCs, thus improving animal survival significantly. As overexpression of SA and acidic tumor microenvironment are common features of tumors, 5-BPA molecule as a ligand will have broad application for diagnosis and therapy of cancer and other applications in the field of bioengineering. Furthermore, the ability of our boronic acid-conjugated polymer systems for inducing T cell proliferation suggest the prospective of such synthetic lectin to induce proliferation of T cells *in vitro*. We perceive the potential of developing *in vitro* culture systems coated with boronic acid conjugated polymer which can lead to more efficient and cost-effective technique for culturing T cells, as well as potential for developing novel immunotherapeutic approaches against cancer. According to the referee's comments, this dissertation is eligible for applying for a diploma of Ph.D. (engineering).