**Original Article**

**N-Propionyl polysialic acid precursor enhances the susceptibility of multiple myeloma to antitumor effect of anti-NprPSA monoclonal antibody**

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**Aim:** To study the antitumor effect of anti-NprPSA monoclonal antibody (mAb) in combination with ManNPr, a precursor of N-propionyl PSA, in multiple myeloma (MM), and to explore the mechanisms of the action.

**Methods:** Human multiple myeloma cell line RPMI-8226 was tested. The cells were pre-treated with ManNPr (1, 2, and 4 mg/mL), and then incubated with anti-NprPSA mAb (1 mg/mL). Cell apoptosis *in vitro* was detected using MTT assay and flow cytometry. BALB/c nude mice were inoculated sc with RPC5.4 cells. On 5 d after the injection, the mice were administered sc with anti-NprPSA mAb (200 μg/d) and ManNPr (5 mg/d) for 8 d. The tumor size and body weight were monitored twice per week. TUNEL assay was used for detecting apoptosis *in vivo*. The apoptotic pathway involved was examined using Western blot analysis and caspase inhibitor.

**Results:** Treatment of RPMI-8226 cells with anti-NprPSA mAb alone failed to inhibit cell growth *in vitro*. In RPMI-8226 cells pretreated with ManNPr, however, the mAb significantly inhibited the cell proliferation, decreased the viability, and induced apoptosis, which was associated with cleavage of caspase-3, caspase-8, caspase-9, and poly(ADP-ribose) polymerase. In the mouse xenograft model, treatment with the mAb in combination with ManNPr significantly inhibited the tumor growth, and induced significant apoptosis as compared to treatment with the mAb alone. Moreover, apoptosis induced by the mAb *in vivo* resulted from the activation of the caspases and poly(ADP-ribose) polymerase.

**Conclusion:** The anti-NprPSA mAb in combination with ManNPr is an effective treatment for *in vitro* and *in vivo* induction of apoptosis in multiple myeloma.

**Keywords:** multiple myeloma; monoclonal antibody; N-propionyl polysialic acid; apoptosis; caspases

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**Introduction**

Sialic acids are the most ubiquitous sugars found in eukaryotic cells. They reside primarily in terminal positions of cell surface glycoconjugates, where they play critical roles in biological events such as cell-cell recognition, migration and homing, and protein stability. Sialic acids can also serve as substrates for infectious agents. Polysialic acid (PSA), a linear homopolymer composed of α-(2-8)-linked N-acetyl-neuraminic acid (NeuAc) residues, is a unique biological form of sialic acid that is an important cancer-associated antigen. Several studies have taken advantage of the permissiveness of the sialic acid and PSA biosynthetic pathways to remodel the cell-surface landscape of tumors both *in vitro* and *in vivo*. This remodeling can be performed by replacing N-acetyl-mannosamine (ManAc), the physiological precursor of sialic acid, with an exogenous source of unnatural N-acyl mannosamines, which results in the introduction of these unnatural sialosides into surface glycoconjugates. This biochemical engineering, when applied to different cell systems, has so far revealed several important biological functions of the N-acyl side chain of sialic acid. The treatment of lymphoma cells with ManNPr reduced their infectibility by several sialic acid-dependent viruses, *e.g.*, influenza A virus. Human diploid lung fibroblasts displayed a loss of density-dependent growth control after biochemical engineering. Liu *et al.* have reported that poorly immunogenic PSA on the surface of RMA leukemia cells can be biochemically engineered to express N-propionyl PSA by using ManNPr as a precursor and that the resultant cells became susceptible to treatment with an N-propionyl PSA-specific monoclonal antibody *in vitro* and *in vivo*. In this study, we have extended the same strategy to human multiple myeloma (MM) cells.

MM accounts for approximately 10% of malignant hema-
tologic neoplasms and is resistant to conventional chemotherapy, high-dose radiotherapy, autologous stem cell transplantation, and allogeneic transplantation\[^{8-9}\]. A promising alternative strategy is the development of specific immunotherapies selectively targeting myeloma cells\[^{10-13}\]. However, a major problem in this area is the immune tolerance to tumor cells and tumor-associated antigens\[^{14, 15}\]. To overcome this problem, this study examined the potential of improving the antigenicity of myeloma through metabolic engineering of its cell surface carbohydrate antigens. N-acetyl-poly sialic acid (NAcPSA), the most prominent sialic acid in eukaryotes, is overexpressed on multiple myeloma (MM) cells and is closely related to the poor prognosis of MM patients\[^{16}\]. Therefore, we overexpressed N-propionyl PSA expressed on the surface of MM cells by using ManNPr as a precursor may be a potential target for the treatment of multiple myeloma. In this study, the anticancer effects of an N-propionyl PSA-specific monoclonal antibody in MM have been extensively investigated \textit{in vitro} and in a mouse xenograft model.

**Materials and methods**

**Chemicals and reagents**

ManNPr and NprPSA-keyhole limpet hemocyanin (NprPSA-KLH) were obtained from the Second Military Medical University (Shanghai, China). Anti-human β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-human poly(ADP-ribose) polymerase (PARP), anti-human caspase-3, anti-human caspase-8, anti-human caspase-9, and all secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Cell culture**

The human multiple myeloma cell line RPMI-8226 was obtained from the Shanghai Cancer Institute (Shanghai, China). The cell lines were maintained in suspension culture using RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), 100 units/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO\(_2\).

**Monoclonal antibody production**

Murine mAbs to NprPSA-KLH were prepared by standard methods according to Plested \textit{et al}\[^{17}\]. Briefly, BALB/c mice were immunized four times intraperitoneally, followed by one intravenous injection without adjuvant. Hybridomas were prepared by the fusion of spleen cells with SP 2/0 as described. Putative hybridomas secreting NprPSA-specific antibodies were selected by ELISA using NprPSA-KLH as a coating antigen. The immunoglobulin class and subclass were also determined as IgG1 by ELISA. The mAb used in this study was purified by protein G affinity chromatography (Pierce, Rockford, USA). The potency of the antibody in ascites was 5×10\(^4\).

**Analysis of cell growth inhibition**

The effect of the anti-NprPSA mAb on cell proliferation was measured using an MTT-based assay. Briefly, 1×10\(^6\) cells were pretreated with ManNPr (0, 1, 2, and 4 mg/mL) in 24-well plates. Tumor cells (1×10\(^5\)–2×10\(^5\)), after treatment with ManNPr, were harvested, washed with PBS, and incubated with anti-NprPSA mAb (1 mg/mL) on ice for 1 h. The cells were washed and incubated with 10% rabbit complement (Cedarlane Laboratories, Ontario, Canada) at 37°C for 2 h. Thereafter, 10 μL of MTT solution (5 mg/mL in PBS) was added to each well and then incubated for 4 h. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved in 0.15 mL of DMSO, and then the optical density (OD) value was measured at 570 nm by a microplate reader. The following formula was used: cell proliferation inhibited (%)=[1–(OD of the experimental samples/OD of the control)]×100%.

**Flow cytometry analysis of apoptotic cell population**

To determine the level of apoptosis, anti-NprPSA mAb and rabbit complement-treated cells, after incubation with ManNPr, were washed in PBS and resuspended in binding buffer at a concentration of 1×10\(^6\) cells/L. A total of 195 μL of the solution was transferred to a 5 mL culture tube with 5 μL annexin V-FITC (BD, USA) added. The tube was then incubated for 30 min at room temperature in the dark. The cells were washed with binding buffer and resuspended in 190 μL binding buffer with 10 μL PI added. Finally, the tube was gently vortexed and incubated for another 30 min in the dark. The cells were analyzed by a flow cytometer.

**Western blot analysis**

RIPA buffer in the presence of a protease inhibitor cocktail (Roche) was used to extract total protein. The lysate was centrifuged at 50,000×g for 10 min at 4°C to remove insoluble material. Cytosolic protein without mitochondrial protein was extracted using the Proteo Extract Cytosol/Mitochondria Fractionation kit (Calbiochem) according to the manufacturer’s instructions. The protein content was determined using a protein assay kit (Bio-Rad). The supernatant (30 μg protein) was subjected to 8%–15% SDS-PAGE electrophoresis. Proteins were electroblotted onto nitrocellulose membranes. After blocking with 5% nonfat milk for 1 h, the blots were probed with primary antibodies overnight at 4°C. The blots were then incubated with HRP-conjugated anti-IgG for 2 h. After washing, the blots were developed using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

**Antitumor effect of anti-NprPSA mAb to mouse MM model**

Four-week-old BALB/c nude mice were purchased from the Shanghai Animal Center (Shanghai, China). The mice were inoculated subcutaneously (sc) with RPC5.4 cells (10\(^6\) cells/mouse) obtained from the Shanghai Cancer Institute, and 5 d after injection, they were inoculated sc daily with anti-NprPSA mAb (200 μg/mouse) and precursor ManNPr (5 mg/mouse) for a period of 8 d. The tumor size and body
weight of the mice were monitored twice per week. The mice were weighed, and tumor volumes were assessed by measuring the two perpendicular dimensions using calipers and the formula \((a \times b)/2\), where \(a\) is the larger and \(b\) is the smaller dimension of the tumor. When treatment was finished, the mice were sacrificed, and the tumors were excised. Tumor tissues were trimmed of extraneous fat or connective tissue, homogenized in RIPA buffer (100 mg tumor tissue/1 mL RIPA) and prepared for Western blot analysis.

**TUNEL analysis**

Cell apoptosis in vivo was investigated using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer’s instructions (Promega, USA). Three tumor nodules per group in the sc tumor model were analyzed after the last treatment.

**Statistical analysis**

Statistical analysis was performed using the unpaired Student’s t-test and an analysis of variance (one-way ANOVA). The data were presented as mean±SD of at least three independent experiments. The accepted level of significance was \(P<0.05\).

**Results**

**Anti-NprPSA mAb inhibited myeloma cell proliferation**

We first determined the effect of the anti-NprPSA mAb on the growth of MM cell lines using the MTT assay, and the results are shown in Figure 1A and 1B. Following pre-treatment with the precursor (ManNPr), RPMI-8226 cells were further treated with anti-NprPSA mAb and rabbit complement at 37°C. The resultant cell counts demonstrated that the inhibition of tumor cell proliferation was dependent only on the time and dose of their exposure to ManNPr because anti-NprPSA mAb alone failed to inhibit cell growth. Thus, an increase in NPr polysialic acid expression on the cell surface led to an increase in the inhibition of cell proliferation (Figure 1A). The inhibition of tumor cell proliferation by rabbit complement alone was not significant, thus indicating that the proliferation of these cells is controlled by the specificity of the antibody used (Figure 1B).

**Anti-NprPSA mAb induced apoptosis of myeloma cells**

A decrease in myeloma cell proliferation may also result from the induction of apoptosis, so we increased NPr polysialic acid expression on the cell surface. This led to an increase in the inhibition of cell proliferation (Figure 1A). The inhibition of tumor cell proliferation by rabbit complement alone was not significant, thus indicating that the proliferation of these cells is controlled by the specificity of the antibody used (Figure 1B).

**Anti-NprPSA mAb inhibited tumor growth in an animal model of myeloma**

To determine whether anti-NprPSA mAb could control tumor growth in vivo, we established a subcutaneous mouse xenograft model. Tumor growth was routinely monitored by measurement of tumor size. The data showed that in combination with ManNPr, anti-NprPSA mAb had a greater effect on tumor size than anti-NprPSA mAb alone, and the group treated with ManNPr and mAb resulted in a significantly increased regression of established tumors compared with

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**Figure 1.** Antibody-mediated inhibition is dependent on the expression of NPr polysialic acid in tumor cells. (A) RPMI-8226 cells were incubated with increasing concentrations of ManNPr for 3 d. At daily intervals, the cells were harvested, washed with PBS, and incubated with the anti-NprPSA mAb, as described previously. The cells were then subjected to an MTT assay. Data were representative of values from at least three independent experiments. (B) RPMI-8226 cells were incubated with ManNPr (4 mg/mL), and aliquots were harvested at different time intervals. They were then washed with PBS and incubated with or without the anti-NprPSA mAb and subjected to the MTT assay, as described previously. Data were representative of values from at least three independent experiments.
the group treated with mAb alone or the control group (Figure 3A). At the end of the experiment, mice were sacrificed and the tumors were excised from the body. The average tumor weights in ManNPr and anti-NprPSA mAb treated group were 0.18±0.07 g, which were significantly lower compared to the 0.74±0.12 g tumor weight average in the control group (**P**<0.05; Figure 3B).

**Anti-NprPSA mAb can induce myeloma cells apoptosis in vivo**

To test whether apoptosis was the mechanism by which anti-NprPSA mAb produced anti-tumor effects in vivo, we performed a TUNEL assay on histological sections. As shown in Figure 4, there were more apoptotic cells (with green nuclei) in tumor tissues from the ManNPr and anti-NprPSA mAb treated mice than from the control treated mice (**P**<0.05).

To validate the mechanism by which ManNPr and anti-NprPSA mAb exert their antitumor effect, we investigated the in vivo expression of some key apoptosis-related proteins examined in the in vitro assay. The expression trends of pro-caspases and the precursor of PARP were all in agreement with the in vitro studies (Figure 5).

**Discussion**

Targeting cell-surface antigens using monoclonal antibodies represents a very attractive approach to cure multiple myeloma[18–21]. However, a major problem that has hindered further progress in the area is immune tolerance. Recently, cell glycoengineering has been established as a very useful technique for the modification of carbohydrate antigens on the cell surface[22]. By biochemically engineering cell surface antigens, we are able to temporarily remodel the cell surface and render it susceptible to targeted antibody responses. In this study, we found that multiple myeloma cells pre-treated with the precursor ManNPr exhibit a significant reduction in cell viability and proliferation using the mAb specific for NPr polysialic acid. Furthermore, an increase in NPr polysialic acid expression on the cell surface led to an increase in the inhibition of cell proliferation. As mentioned in a previous study, Liu et al[3] found that a precursor (ManNPr), when incubated with leukemic cell lines RBL-2H3 and RMA, resulted in
expression of the altered α2-8 N-propionylpolysialic acid on the surface of tumor cells and induced their susceptibility to cell death mediated by monoclonal antibody 13D9, which specifically recognizes α2-8 N-propionylated polysialic acid.

We investigated whether the apoptotic pathway is involved in the inhibition of cell proliferation caused by the anti-NprPSA mAb. Flow cytometric analysis showed that the anti-NprPSA mAb and complement treatment significantly increased the number of apoptotic cells compared to treatment with complement alone. Exposure to the mAb and complement decreased expression of the PARP precursor and increased caspase-3, caspase-8, and caspase-9 activities in RPMI-8226 cells, which suggested that the mechanism of apoptosis was involved the caspase-dependent apoptotic pathway. This result was supported by other data showing that pre-treatment with the pan-caspase inhibitor z-VAD-fmk decreased the apoptosis induced by anti-NprPSA mAb. Although the mAb’s mechanism of action that initiates and induces tumor cell death is not entirely known so far, it has been proposed that mAbs are able to bind and cross-link target molecules, subsequently eliciting antibody-dependent cell-mediated cytotoxicity (ADCC), activating complement-dependent cytotoxicity (CDC), and/or directly inducing tumor cell apoptosis[23, 24]. For the induction of mAb-mediated ADCC, the binding of the Fc portion of mAbs to Fcγ receptors on immune cells is necessary. To induce antibody-mediated CDC, the cross-linking of mAbs activates complement cascades, which trigger the assembly of the membrane attack complex and subsequent osmotic cell lysis. Moreover, a few mAbs can directly induce tumor cell apoptosis through transduction of an apop-
apoptotic signal to cells, which triggers intracellular apoptotic signal pathways and cleaves caspases and poly(ADP-ribose) polymerase (PARP), leading to tumor cell apoptosis.

Having observed that apoptosis occurs in RPMI-8226 cells incorporating ManNPr into the cell surface treated with the anti-NprPSA mAb, we wanted to determine whether the anti-NprPSA mAb induces multiple myeloma cell apoptosis in an in vivo system. Following pre-treatment with ManNPr, the mAb inhibited tumor growth. Histology of TUNEL-stained tumor sections showed that ManNPr and mAb treatment was able to induce statistically significant apoptosis (P<0.05) compared to control treatments. In the in vivo assay, the expression trend of the caspases and PARP were all in agreement with the in vitro studies. These results indicated that apoptosis is the main mode of death of multiple myeloma cells treated with the ManNPr precursor and the anti-NprPSA mAb.

In summary, our results indicate that the anti-NprPSA mAb can decrease tumor growth by binding modified surface antigens on myeloma cells and promoting apoptosis in vitro and in a multiple myeloma mouse xenograft model. Evidence presented in our study suggests that the anti-NprPSA mAb may have utility as a component of a clinical regimen for myeloma malignancies.

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Author contribution
Yu-hua CHEN and Yi DING designed the research; Hong XIONG performed the research and wrote the paper; and Ai-bin LIANG, Bing XIU, and Jian-fei FU revised the paper.

References
1. Kepler OT, Stehling P, Herrmann M, Kayser H, Grunow D, Reutter W, et al. Biosynthetic modulation of sialic acid-dependent virusreceptor interactions of two primate polyoma viruses. J Biol Chem 1995; 270: 1308–14.
2. Daniel L, Durbec P, Gautherot E, Rouvier E, Rougon G, Figarella-Branger D. A nude mice model of human rhabdomyosarcoma lung metastases for evaluating the role of polysialic acids in the metastatic process. Oncogene 2001; 20: 997–1004.
3. Liu T, Guo Z, Yang Q, Sad S, Jennings HJ. Biochemical engineering of surface alpha 2-8 polysialic acid for immunotargeting tumor cells. J Biol Chem 2000; 275: 32832–6.
4. Zou W, Borrelli S, Gilbert M, Liu T, Pon RA, Jennings HJ. Bioengineering of surface GD3 ganglioside for immunotargeting human melanoma cells. J Biol Chem 2004; 279: 25390–9.
5. Prescher JA, Dube DH, Bertozzi CR. Chemical remodelling of cell surfaces in living animals. Nature 2004; 430: 873–7.
6. Kepler OT, Horstkorte R, Pawlita M, Schmidt C, Reutter W. Biochemical engineering of the N-acetyl side chain of sialic acid: biological implications. Glycobiology 2001; 11: 11R–18R.
7. Wieser JR, Heisner A, Stehling P, Oesch F, Reutter W. In vivo modulated N-acyl side chain of N-acetylleucaminic acid modulates the cell contact-dependent inhibition of growth. FEBS Lett 1996; 395: 170–3.
8. Rajkumar SV, Gertz MA, Kyle RA, Greipp PR; Mayo Clinic Myeloma, Amyloid, and Dysproteinemia Group. Current therapy for multiple myeloma. Mayo Clin Proc 2002; 77: 813–22.
9. Kyle RA, Rajkumar SV. Multiple myeloma. Blood 2008; 111: 2962–72.
10. Recigno M, Avogadri F, Curigliano G. Challenges and prospects of immunotherapy as cancer treatment. Biochim Biophys Acta 2007; 1776: 108–23.
11. Bladé J, de Larrea CF, Rosiñol L. Incorporating monoclonal antibodies into the therapy of multiple myeloma. J Clin Oncol 2012; 30: 1904–6.
12. Hosen N, Ichihara H, Mugitani A, Aoyama Y, Fukuda Y, Kishida S, et al. CD48 as a novel molecular target for antibody therapy in multiple myeloma. Br J Haematol 2012; 156: 213–24.
13. Tai YT, Anderson KC. Antibody-based therapies in multiple myeloma. Bone Marrow Res 2011; 2011: 924058.
14. Sharabi A, Ghera NH. Breaking tolerance in a mouse model of multiple myeloma by chemoimmunotherapy. Adv Cancer Res 2010; 107: 1–37.
15. Han S, Wang B, Cotter MJ, Yang L, Zucai J, Moreb JS, et al. Overcoming immune tolerance against multiple myeloma with lentiviral calnexin-engineered dendritic cells. Mol Ther 2008; 16: 269–79.
16. Bily R, Tomin A, Mahorivska I, Shalay O, Lohinskyy V, Stoika R, et al. Antibody-mediated sialidase activity in blood serum of patients with multiple myeloma. J Mol Recognit 2011; 24: 576–84.
17. Piester JS, Makepeace K, Jennings MP, Gidney MA, Lacelle S, Brisson J, et al. Conservation and accessibility of an inner core lipopolysaccharide epitope of Neisseria meningitidis. Infect Immun 1999; 67: 5417–26.
18. Yang J, Yi Q. Therapeutic monoclonal antibodies for multiple myeloma: an update and future perspectives. Am J Blood Res 2011; 1: 22–33.
19. van de Donk NW, Kamps S, Mutis T, Lokhorst HM. Monoclonal antibody-based therapy as a new treatment strategy in multiple myeloma. Leukemia 2012; 26: 199–213.
20. Cao Y, Lan Y, Qian J, Zheng Y, Hong S, Li H, et al. Targeting cell surface β2-microglobulin by pentameric IgM antibodies. Br J Haematol 2011; 154: 111–21.
21. Richardson PG, Loniol S, Jakubowiak AJ, Harousseau JL, Anderson KC. Monoclonal antibodies in the treatment of multiple myeloma. Br J Haematol 2011; doi: 10.1111/j.1365-2141.2011.08790.x.
22. Pan Y, Chefalo P, Nagy N, Harding C, Guo Z. Synthesis and immunological properties of N-modified GM3 as therapeutic cancer vaccines. J Med Chem 2005; 48: 875–83.
23. Bello C, Sotomayor EM. Monoclonal antibodies for B-cell lymphomas: rituximab and beyond. Hematology Am Soc Hematol Educ Program 2007; 233–42.
24. Yang J, Yi Q. Therapeutic monoclonal antibodies for multiple myeloma: an update and future perspectives. Am J Blood Res 2011; 1: 22–33.