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Targeted depletion of tumour-associated macrophages by an alendronate–glucomannan conjugate for cancer immunotherapy

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ABSTRACT

Tumour-associated macrophages (TAMs) are a set of macrophages residing in the tumour microenvironment. They play essential roles in mediating tumour angiogenesis, metastasis and immune evasion. Delivery of therapeutic agents to eliminate TAMs can be a promising strategy for cancer immunotherapy but an efficient vehicle to target these cells is still in pressing need. In this study, we developed a bisphosphonate–glucomannan conjugate that could efficiently target and specifically eliminate TAMs in the tumour microenvironment. We employed the polysaccharide from *Bletilla striata* (BSP), a glucomannan affinitive for macrophages that express abundant mannose receptors, to conjugate alendronate (ALN), a bisphosphonate compound with *in vitro* macrophage-inhibiting activities. In both *in vitro* and *in vivo* tests, the prepared ALN-BSP conjugate could preferentially accumulate in macrophages and induced them into apoptosis. In the subcutaneous S180 tumour-bearing mice model, the treatment using ALN-BSP effectively eliminated TAMs, remarkably inhibited angiogenesis, recovered local immune surveillance, and eventually suppressed tumour progression, without eliciting any unwanted effect such as systematic immune response. Interestingly, ALN alone failed to exhibit any anti-TAM activity *in vivo*, probably because this compound was susceptible to the mildly acidic tumour microenvironment. Taken together, these results demonstrate the potential of ALN-BSP as a safe and efficient tool targeted at direct depletion of TAMs for cancer immunotherapy.

1. Introduction

Tumour-associated macrophages (TAMs) are a set of macrophages residing in the tumour microenvironment [1–3]. They play pivotal roles in the multiple stages of tumour development and progression. In particular, they secrete a plethora of cytokines that induce cell migration, mediate immunosuppression and promote angiogenesis [3,4]. Delivery of therapeutic agents to eliminate TAMs, thereby inhibiting angiogenesis and cancer metastasis, has emerged as a promising strategy for the treatment of cancer [4–7].

Targeted delivery of drugs to TAMs is challenging. These cells, scattered in the extremely complex tumour microenvironment, are surrounded by numerous other cell types and blood capillaries [1,3,8]. To target these cells, a feasible strategy is to devise drug carriers that can be recognised by specific receptors on TAMs. Based on their different surface receptor patterns and other phenotypes, macrophages are generally categorised into two types of 'polarisation': one is inflammatory (M1, or 'classically activated') and the other non-inflammatory (M2, or 'alternatively activated') [2]. TAMs are considered as M2-polarised macrophages that abundantly express M2-specific surface markers including the macrophage mannose receptor (MMR; CD206 in humans or CD205 in mice) [2,9]. Recently, MMR is suggested to be a desirable 'gateway' in mediating drug delivery to macrophages. It contains C-type carbohydrate recognition domain (CRD) that binds ligands such as mannose-ending saccharides, and efficiently mediates internalisation of the ligands, without exerting unwanted intracellular events [10,11].

Two major strategies are available to utilise MMR for macrophage targeting. One is to modify the mannose moieties on the surface of nanoparticles [12,13], liposomes [14] or polymeric micelles [15], for delivery of nucleic acids to alveolar macrophages [12] or imaging agents/vaccines to TAMs [13,14]. Alternatively,

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mannose-containing polymers with affinity for MMR could act as vehicles in their own right. For example, in our previous studies, we derived and characterised a glucomannan polysaccharide from the natural herb *Bletilla striata* (BSP) [16–18], and established its affinity for MMR and its capability in mediating therapeutic oligonucleotides into the cultured macrophage cell lines [17] – though its capability to target TAMs *in vivo* has yet to be tested.

In this study, we aimed to employ BSP to conjugate alendronate (4-amino-1-hydroxybutylidene 1,1-bisphosphonate, ALN) for the targeted delivery of ALN to the TAM population. We hypothesised that BSP, the polysaccharide vehicle with binding affinity for MMR, would play an essential role in directing ALN to target and then eliminate TAMs in the tumour microenvironment. We set out to examine the efficiency of ALN-BSP in targeting and eliminating macrophages both *in vitro* and *in vivo*, as well as its efficacy in inhibiting tumour progression in a mouse tumour model.

2. Materials and methods

2.1. Materials

The dry herb of *Bletilla striata* was purchased from China Pharmaceutical Corporation-Canton (Guangzhou, China). Fluorescein-5-thiosemicarbazide (5-FTSC) and 5-carboxyfluorescein (5-FAM) were purchased from AnaSpec (USA). The beta-actin (No. 4967) and anti-rabbit IgG-HRP (No. 7074) antibodies were purchased from Cell Signalling Technology (USA). Anti-Rap 1A (sc-1482), bovine anti-goat IgG-HRP (sc-2378) and goat anti-mouse IgG-HRP (sc-2005) antibodies were provided by Santa Cruz (USA). Anti-mannose receptor antibody (ab8918) was provided by Abcam (UK). The recombinant human macrophage mannose receptor (MMR) was purchased from R&D Systems (USA). General chemicals and reagents were from Sigma–Aldrich unless otherwise stated.

Mouse macrophage cell line Raw 264.7 (ATCC TIB-71), mouse sarcoma cells (S180, ATCC CCL-8), human umbilical vein endothelial cells (HUVECs, ATCC CRL-1730), and human lung carcinoma A549 cell line (ATCC CCl-185) were obtained from the American Type Culture Collection (ATCC, USA). Raw 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS); both HUVEC and A549 cells maintained in RPMI 1640 medium with 10% FBS. All reagents used for cell culture were purchased from Gibco/Life Technologies (USA).

2.2. Preparation of BSP-conjugated alendronate (ALN-BSP)

The glucomannan, BSP, was routinely purified and characterised by our two groups following our in-house protocol reported previously [16–18]. Briefly, the polysaccharide component was extracted from the pre-homogenised dry herb in boiling water and then precipitated by three volumes of cold ethanol (4 °C) for overnight. After repeated ethanol wash, the precipitation was re-dissolved in deionised (DI) water, dialysed against membrane (Mw cut-off 3500 Da) and lyophilised to obtain the crude BSP for storage in aliquot. The crude sample further underwent a pre-optimised procedure comprising serial assays including Sevag's assay (chloroform/n-butanol, 4:1, v/v), ion-exchange chromatography (DEAE cellulose, 3 × 45 cm) and gel-filtration chromatography (Sephadex G-200, 2 × 150 cm, GE Amersham, Sweden) to produce pure BSP glucomannan [16,17]. The purity of BSP was examined by anthrone test, elemental analysis as well as high performance liquid chromatography (HPLC).

Preparation of the ALN-BSP conjugates comprises two steps: 1) oxidise the adjacent hydroxyl groups on the BSP chain to aldehydes; and 2) link the aldehydes and amines of ALN to form imines (Schiff base) that are further reduced to amides in the final product of ALN-BSP [19–21] (Fig. 1A). For Step 1, we added sodium periodate ($NaIO_4$, 200 mg) to the BSP solution (w/v 2%, in 10 ml DI water) and performed orthogonal tests with three variables – temperature (T), pH and time (t). The optimal condition (45 °C, pH 3.5 and 8 h) was selected for use throughout this study (Table 1). For Step 2, oxidised BSP (w/v 2%, in 5 ml DI water) reacted with alendronate (100 mg) at 80 °C for 15 min under stirring, with three different pH (4.0, 7.0 and 10.0) being compared. The reaction was stopped by addition of sodium borohydride ($NaBH_4$, 100 mg) for 5 min, followed by dialysis (Mw cut-off 3500 Da) and lyophilisation for collection of the ALN-BSP product. In some experiments, 5-FTSC was co-conjugated with ALN to ox-BSP to form ALN-BSP-FTSC for fluorescent visualisation.

2.3. Characterisation of ALN-BSP

Together with BSP, the prepared ALN-BSP samples were characterised in several aspects. Firstly, their purity was examined by GPC-ELSD with TSK-GEL G4000 PWXL (TOSOH, Japan). This HPLC system consist of a Waters e2695 platform (Waters, USA) linked with evaporative light scattering detector (ELSD, Shimadzu, Japan). Their molecular weight was determined by GPC-MALLS-RI. The samples were applied to an Agilent 1100 system equipped with a DAWN EOS multi-angle laser light scattering photometer linked with Optilab rEX refractive index (RI) detector (Wyatt Technology, CA, USA). Secondly, the contents of four elements, carbon (C), hydrogen (H), nitrogen (N) and phosphorus (P), in BSP and ALN-BSP were determined by elemental analysis (Vario MICRO, Elementar, Germany) at the Centre for Modern Analysis, Nanjing University. Thirdly, the addition of ALN to BSP was assessed with ^{32}P Nuclear magnetic resonance (^{32}P NMR, National Centre of Biomedical Analysis, Beijing, China). Fourthly, the morphology and particle size of both BSP and ALN-BSP were analysed through nanoparticle tracking analysis (NTA, Nanosight NS500 System, UK), Zetasizer Nano ZSP instrument (Malvern), as well as transmission electron microscopy (TEM, Hitachi, Guangdong Institute for Microbiology). For the TEM observation, BSP or ALN-BSP samples (1 mg/ml) were placed on a copper grid, dried and stained with phosphotungstic acid, followed by visualisation with an accelerating voltage at 80 kV.

2.4. Drug loading efficiency

The content of alendronate was determined by the molybdenum blue colorimetric assay [22,23]. Briefly, we prepared three buffer solutions – i) Solution 1: 0.16 g ammonium persulphate was dissolved in 4 ml DI water; ii) Solution 2: 0.15 g ammonium molybdate was dissolved in 4 ml diluted sulphuric acid (5 mol/l); iii) Solution 3: 0.01 g paramethylaminophenol sulphate, 0.58 g sodium hydrogen sulphite and 0.02 g sodium sulphite were dissolved in 4 ml DI water. We also prepared solution of alendronate sodium in a series of concentrations (10, 50, 100, 150 and 200 μg/ml) as standard, together with our samples. We added 50 μl of each alendronate standard or undetermined sample, and then 20 μl Solution 1, into a 1.5 ml Eppendorf tube, heated the tube for 30 min at 100 °C, and added 20 μl Solution 2 and 20 μl Solution 3, separately, into the same tube. Finally, we transferred 100 μl of each reaction mixture into a 96-well plate and recorded the absorbance at 690 nm, plotting the standard curve and determining the sample concentrations. The loading efficiency of ALN to BSP was calculated as:

$$\text{Drug loading efficiency (\%)} = (\text{weight of ALN/weight of ALN-BSP}) \times 100\%$$

2.5. MMR-binding assay

The binding between the recombinant human MMR/CD206 and BSP was examined using the mannose–agarose beads assay [24,25]. The beads were incubated with 1) MMR, 2) MMR plus 0.2 or 2 mg/ml mannan, 3) MMR plus 0.2 or 2 mg/ml BSP and 4) saline control at 4 °C for 1 h and then centrifuged at 10,000 rpm for 10 min. The beads in the sediment, containing the bound MMR, were washed with PBS for three times and stained with PE-conjugated anti-human CD206 (BioLegend, USA) at 4 °C for 1 h. After washing for three times with PBS, the beads were re-suspended in PBS and observed with a fluorescent laser microscope (IX73, Olympus Corp, Japan). The amount of unbound MMR in the supernatant was detected by Western blotting.

2.6. Preparation of ALN-FAM

The 5-FAM fluorophore was coupled to ALN by EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide) at room temperature for 24 h. The product was separated using a Sephadex LH-20 column (GE Amersham, Sweden). The samples were analysed with a high-performance liquid chromatography (HPLC, Waters 2695, USA) with UV and fluorescent detectors (Supplementary Fig. 1).

2.7. Cell viability, cell cycle and apoptosis analysis

In all cell biology tests involving ALN, ALN-BSP and BSP, we used the dose of ALN as standard and converted the concentrations of ALN-BSP and BSP to that of ALN. For example, when we used 1 μM ALN, we in parallel used ALN-BSP that contained 1 μM ALN and pure BSP that would conjugate 1 μM ALN in the ALN-BSP conjugate. We refer these doses as 'equivalent doses' throughout this study.

Cell viability was tested in two major sets of experiments. Because ALN had been reported to inhibit macrophage growth *in vitro*, we firstly tested how different doses of ALN, ALN-BSP and BSP would affect the growth of Raw 264.7 macrophages. In this experiment, cells were seeded in 96-well plates and incubated for 24 h before treatment with ALN, ALN-BSP or BSP at various concentrations (0.1, 1, 10 and 100 μM; equivalent doses applied) for another 24 h. We next examined whether the three substances – in a dose equivalent to 100 μM of ALN – could exhibit different activities on different cells and in varying pH conditions. For this test, Raw 264.7, A549 and HUVEC were seeded in 96-well plates for 24 h, before addition of three different samples at various pH (6.5, 7.0, 7.5 and 8.0) for another 24 h.

After these treatments, the cell viability was examined mainly with MTT assay and in part with BrdU proliferation assay. For MTT assay, to each well 10 μl working solution of MTT (5 mg/ml) was added and incubated for 4 h, followed by DMSO dissolution and absorbance reading at 490/650 nm. For BrdU assay, the procedure was strictly following the manufacturer's protocol (BrdU Cell Proliferation ELISA-colorimetric, Roche, Switzerland). For both assays, the inhibitory ratio of each sample group was calculated and normalised to the control group.