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Development of novel anti-CD19 antibody-drug conjugates for B-cell lymphoma treatment

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ABSTRACT

B-cell lymphoma remains one of the most refractory tumors, and as such the development of novel treatment approaches, such as antibody-drug conjugates (ADCs), is required. To improve the stability and homogeneity of the ADCs, a humanized anti-CD19 monoclonal antibody (RC58) was developed in the present study. RC58 was based on the CD19 antigen as a potential molecular target of human B-cell lymphomas. RC58 has high CD19-binding affinity and can be internalized in CD19-positive cells through endocytosis. Furthermore, three types of RC58-based ADCs (ADC-1, ADC-2, and ADC-3) were generated using three kinds of Maleimide-PEG-based linkers with two different cytotoxins. The anti-tumor activities of the ADCs were confirmed by *in vitro* and *in vivo* experiments. The stability of the ADCs was also evaluated by incubation in human plasma for 10 days. *In vitro* experiments showed that the three ADCs had distinct inhibitory effects on several B-lymphoma cell lines. Meanwhile, a close correlation between efficacy and drug concentration was found in a nude mouse xenograft model of human B-cell lymphoma, after treatment with RC58-based ADCs. Our results suggest that ADC-1, with high efficiency, could be used as a potential therapeutic agent for human B-cell malignancies.

1. Introduction

B-cell lymphomas are types of hematological malignancies that begin in B lymphocytes and are much more common than T-cell lymphomas. Although most patients that suffer from B-cell lymphomas respond to the current first line therapeutic treatment, the tumor recurrence rate in those patients is still high and their prognosis is poor. Therefore B-cell lymphomas remain one of the most refractory tumors, and the development of novel treatment approaches is required [1].

CD19 is classified as a type I transmembrane glycoprotein, which is distributed on the surface of B-lymphocytes and follicular dendritic cells, and belongs to the immunoglobulin super family (IgSF) [2]. It plays a vital role by generating activation signals to regulate the process of the B cell development, proliferation and differentiation [3–5]. Moreover, CD19 also plays a key role in B-cell malignancies and autoimmune diseases. CD19 expression has been found in many B-cell lymphoma cells. For example, CD19 monoclonal antibodies (mAbs) were used for recognizing Raji (B-lymphoma cells) and Nalm-6 (pre-B cells) and the adoptive immunotherapy of B cell malignancies [6, 7]. Therefore, CD19 is considered an important biomarker for B-cell malignances.

In recent years, CD19 has been the center of attention in the development of immunotherapy based on molecular target drugs against B-cell lymphomas [8]. Currently, many anti-CD19 therapeutic strategies have been developed, including mAbs, bispecific antibodies [9], and antibody-drug conjugates (ADCs) [10]. Among these strategies, ADCs have gained significant prominence, due to both the toxicity and specificity, resulting from the conjugation of cytotoxic molecules to antibodies targeted against the tumor cells. The ADC mechanism of action involves the specific antibody binding to the antigen on the target cell surface, at which point the ADC-receptor complexes are rapidly internalized and release active toxin within lysosomes, thereby killing tumor cells [11–13]. Several anti-CD19 mAb-based ADCs are currently being tested in various clinical trials, such as SAR3419 (huB4-DM4). SAR3419 is a CD19-targeting ADC, in which a humanized IgG1anti-CD19 antibody is conjugated to a natural potent cytotoxic drug, maytansine derivative (DM4), *via* a cleavable disulfide bond [14]. It has been reported that in xenograft models with NOD/SCID mice, SAR3419

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was more effective in treating acute lymphioblastic leukemia (ALL) than chemotherapy and rituximab [15]. The positive therapeutic effects of SAR3419 in its phase I and II clinical trials have been reported [16, 17]. In addition, other CD19-targeting ADCs are also in various stages of clinical trials. These ADCs include SGN-CD19 (a humanized anti-CD19 antibody conjugated to monomethyl auristatin F, which is a potent synthetic cytotoxic drug), MDX1206 (a humanized anti-CD19 antibody conjugated to pro-drug of duocarmycin SA), and others [18]. Nevertheless, few CD19-targeting ADCs have entered Phase III clinical trials and are currently available for therapeutic use in B-cell lymphoma patients.

Although considerable advances have been made, as to the CD19 antibody-based therapeutic strategies, some stringent safety measures and technical challenges remain, including the design of ADCs to improve the stability and homogeneity of the ADCs. Since one of the biggest challenges in the development of ADCs is the application of suitable linkers for conjugating drugs to antibodies, a novel humanized anti-CD19 antibody, called RC58, was developed in our study. RC58 is attached *via* three Maleimide-PEG-based linkers to different cytotoxins. In the present study, we investigated the characteristics of the RC58 antibody and evaluated the activity of RC58-based ADCs using *in vitro* and *in vivo* studies, as well as the conjugate stability of these ADCs.

2. Materials and methods

2.1. Cell lines

The cell lines used in the present study were obtained from American Type Culture Collection (ATCC, Manassa, VA, USA), and included dihydrofolate reductase (DHFR)-deficient stains of the Chinese hamster ovary cell line (CHO/dhfr-), the mouse myeloma cell line P3X63Ag8, the human HEK-293 cell line, the human Burkitt's lymphoma cell line (Raji), the human diffuse large B-cell lymphoma cell line (HT), the human precursor B-cell leukemia cell line (Nalm-6, CA46 and Jurkat). All cell lines were cultured with RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37 °C.

2.2. Reagents

The linkers and cytotoxins for generating ADCs in this study were obtained from Levena Biopharma (Nanjing, China). The following reagents used for *in vitro* and *in vivo* studies were purchased from different vendors: SMART RACE cDNA kit from Clontech Laboratories (Mountain View, CA, USA); NuPAGE pre-cast gels from Thermo Fisher Scientific (Waltham, MA, USA); Cell Counting Kit-8 (CCK-8) from DOJINDO (Kumamoto, Japan); pHAb Amine Reactive Dyes from Promega (Madison, WI, USA); Fluorescein (FITC) AffiniPure Goat Anti-Human IgG (Fcγ fragment specific) from Jackson Immunoresearch Laboratories (West Grove, PA, USA); and rituximab from Roche (San Francisco, CA, USA). All other chemicals and reagents were of the highest grade available and obtained from Sigma-Aldrich (Santa Clara, CA, USA).

2.3. Animals

BALB/cA nude mice (female, 6–8 weeks old) were obtained from Changzhou Cavens Laboratory Animal Co. Ltd. (Changzhou, China). Experimental mice were housed in pathogen-free conditions with free access to food and water in the animal facility of Simcere (Yantai, China). Animal study protocols were approved by the Animal Care and Use Committee of Simcere. All animal studies were carried out in accordance with institutional guidelines.

2.4. Generation of a murine anti-CD19 mAb

Mice were immunized by intra-peritoneal injection of recombinant human CD19 protein (0.25 mL, 50–100 μg) mixed with incomplete Freund's adjuvant [19]. To generate the mAb, the spleen cells of immunized mice were collected and further fused to a myeloma cell line P3X63Ag8 [20]. The fused cells were diluted and plated into 96-well cell culture plates. The hybridoma supernatant was harvested to test for CD19-binding capacity using an enzyme-linked immunosorbent assay (ELISA). Once the hybridoma cell line that produced the highest titer of anti-CD19 mAb was confirmed, the total RNA was extracted using TRIzol for cDNA synthesis of the variable region in the antibody heavy and light chains using the SMART RACE cDNA kit.

2.5. Construction and screening of the humanized anti-CD19 mAb

In principal, the complementarity-determining regions (CDRs) from the mouse anti-CD19 mAb were grafted into the variable regions of the human antibody, which were then joined to human constant regions, to create a humanized anti-CD19 mAb. Briefly, the murine anti-CD19 mAbs were humanized by transferring the light and heavy chain CDRs into the human IgG1κ light chain and heavy chain framework regions. Through alignment of the sequence with the antibody variable region database, several human IgG1κ framework regions were procured and identified with a high level of homology to the murine anti-CD19 antibody. Subsequently, two different variable region sequences corresponding to each light chain (VL1 and VL2) and heavy chain (VH1 and VH2) were designed. After synthesizing these humanized sequences, each of the two light chain sequences were fused with the constant region of the human IgG1κ by means of polymerase chain reaction (PCR), resulting in two humanized anti-CD19 full-length light chains. Similarly, each of the two humanized heavy chain sequences were also fused with IgG constant regions, resulting in two humanized anti-CD19 full-length heavy chains. The different light and heavy chains were variably combined to generate four types of humanized anti-CD19 antibodies: HZ-1 (VL1-VH1), HZ-2 (VL2-VH1), HZ-3 (VL1-VH2), HZ-4 (VL2-VH2). HEK-293 cells were used for production of the humanized antibodies by transfecting the cells with the relevant heavy and light chain expression vectors. The best version of the humanized anti-CD19 antibody with a high affinity to CD19 antigen was selected for the preparation of ADCs.

2.6. CD19-binding affinity assay

The CD19-binding capacity of different antibodies was measured by ELISA. Briefly, 96-well plates were coated with CD19 recombinant protein (100 μL/per well) at a final concentration of 100 ng/mL at 4 °C overnight. The following day, the coating solution was removed and the plates were blocked with 3% (*v*/v) bovine serum albuminin in phosphate buffer saline (PBS) for 2 h. After washing with PBS, gradient concentrations of anti-CD19 antibodies were added in triplicate. After 2 h of incubation, the plate was washed five times with PBS, followed by the addition of horseradish peroxidase (HRP)-conjugated goat anti-human IgG Fc antibody (100 μL) into each well for an additional 2 h at room temperature. Finally, tetramethylbenzidine (TMB) substrate was added into each well to produce color for visualization. After 8 min of incubation, 100 μL of 2 M H_2SO_4 was used to stop the reaction. The absorbance of each well was read at 450 nm with a plate reader.

2.7. Large scale generation of humanized anti-CD19 antibody

For this purpose, CHO cells (dhfr-) were transfected with a plasmid encoding the heavy and light chain antibody. With a high throughput screening analysis, a high-expression CHO clone was selected and used for large scale generation of the humanized anti-CD19 antibody in a 10-L wave bioreactor using a feed batch process. After 12 days of incubation, the culture broth was collected and clarified using a 0.45-μm filter (Millipore; Boston, MA, USA). Antibody released into the culture broth was purified using Protein A affinity chromatography. Eluted fractions