Bortezomib Enhanced the Efficacy of CAR-T Therapy Through Up-regulating BCMA Expression in Myeloma Cells

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January 19, 2023

Abstract

Multiple myeloma (MM) is a malignant plasma cell disease that causes a large number of deaths every year. In the present study, we investigated the effect of bortezomib, the first-line drug for MM, on B cell maturation antigen (BCMA) of MM cells in order to obtain better efficacy of CAR-T therapy. Flow cytometry and ELISA analysis revealed that the bortezomib steadily increased BCMA expression level in the MM cell line Nalm6-BCMA, thereby enhancing the killing efficiency of CAR-T cells, while the increase of BCMA expression was sustained, allowing more time for CAR-T to effect. In addition, the combination of CAR-T and bortezomib has not yet been developed, suggesting that this combination therapeutic approach may hold great promise for the treatment of MM.

1. Introduction

Multiple myeloma (MM) is a malignant plasma cell disease that is diagnosed in around 34920 people in the US and in approximately 588161 people worldwide each year[1].MM is characterized by the presence of abnormal clonal plasma cells in the bone marrow that cause uncontrolled growth causing destructive bone lesions, kidney injury, anemia, and hypercalcemia. BCMA, also called CD269, is a unique expression molecule on plasma cells, which has been recognized as a great target for new therapeutic options like CAR-T. These years, CAR-T had shown significant efficacy in BCMA-targeted therapy, such as Idecabtagene vicleucel (bb2121, the production of Celgene and Bluebird Bio)[2], and JNJ-4528/ LCAR-B38M[3], etc. However, another problem with these approaches is relapse following BCMA-targeted CAR-T therapy due to antigen loss. Mehmet Kemal Samur et al. (2021) have also pointed out possible causes of BCMA loss such as biallelic loss[4]. Thus, the key to addressing the situation is to improve the expression of BCMA. Additionally, some chemotherapeutic drugs are confirmed to influence the expression of cell surface antigens, this suggests that looking for an appropriate drug to improve the BCMA level is possible.

Bortezomib (BTZ) is a first-line drug for MM, also well-known as Velcade, which was jointly developed by Takeda and Johnson & Johnson and approved by the FDA in 2003 for multiple myeloma. It is a kind of polyhydroxy boric acid that is approved for the inhibitor of protein ubiquitination degradation just like other proteasome inhibitors such as Ixazomib, Carfizomib. The mechanism by which bortezomib promotes apoptosis of cancer cells has been elucidated by researchers that it can cause the up-regulated the cell cycle protein MDM2, lead to stabilization of p53, induction of p21cip/waf-1 and MDM2 expression, an increase in cyclin B and cyclin A, and the activation of cyclin B and cyclin A kinases. G2-M-phase arrest, induction, and an increase in cyclin B1 were G2-M-phase cells to apoptosis[5]. Although bortezomib is so effective in inducing apoptosis of cancer cells that it has become a first-line drug, no attempt has been made to investigate its effect on BCMA.

In this study, we investigated the in-vitro effects of bortezomib on the MM cell line, supporting the hypothesis of the chemotherapeutic drug impacting an antigen expression on cell surface. Our data showed

that bortezomib increased BCMA expression in Nalm6-BCMA cells steadily. It is essential that the Elisa test showed the increased release of two cytokines IFN-γ and TNF-α indicated the killing effect of CAR-T was enhanced. Furthermore, the therapeutic response of CAR-T may be improved in combination with bortezomib.

2. Materials and methods

2.1 Cell culture

The human B lymphoid leukemia cells, Wild-type Nalm-6 and the Nalm-6 BCMA-luc were maintained using RPMI medium 1640(Gibco), supplemented with 10% FBS(PAN, Germany, Cat: ST30-3302) and 1% penicillin-streptomycin (Gibco, Thermo Fisher, Waltham, Massachusetts, Cat: SV30010), at 37 in a humid 5% CO_2 atmosphere. Nalm-6 BCMA cells are Nalm-6 transduced in our laboratory.

2.2 Agents

Bortezomib (Med Chem Express) was dissolved in DMSO and formulated to a concentration of 100μM which was stored at -80, further dissolved to 1μM (working concentration) before use.

2.3 Flow cytometry

We observed BCMA expression changes in two drug treatments with different concentrations (2.5nM and 5nM) over three days. The cells were washed in PBS(Biosharp) and applied with anti-CD269 antibody for 30min. Expression levels were determined by flow cytometry, and three parallel experiments were done at each concentration. (ACEA Biosciences, Inc.)

And then we analyzed that the MFI (Mean Fluorescence Intensity) of 3 flow cytometry samples each group, providing convincing data for research. The MFI relative to 0nM treated group was calculated.

2.4 BCMA-targeted CAR-T Design

2.4.1 Lentiviral production

HEK-293 T cells were used for lentiviral packaging. To achieve the required density for transfection, the density of HEK-293 T cells was amplified to 70%-80% and then using calcium phosphate coprecipitation for transfection. PCLK-based plasmid and the packaging plasmids psPAX2 and pMD2.G (Addgene, Cambridge, Massachusetts) were added in a ratio of 2:2:1. Replacing Media at 12 and 48 hours post transfection, and the 48h and 72h supernatants containing lentiviral particles were collected, concentrated through the ultracentrifugation at 19700 prm, lentivirus was resuspended in DPBS (Gibco S120025) and dispensed, and stored at -80°C until use. A mock lentivirus was produced using an empty PCLK lentivirus plasmid.

2.4.2 Human peripheral blood mononuclear cells (PBMCs) isolation and T cell culture

On day 0, PBMCs were collected from the peripheral blood of healthy donors using cesium chloride density gradient centrifugation and cultured in X-vivo15 (Lonza, Cat: 04-418Q) media supplemented with 5% FBS (PAN, Germany, Cat: ST30-3302) and 100U/mL recombined human IL-2 (rhIL-2, Novoprotein) and with 50U/mL recombined human IL-7/15 (rhIL-7/15, Novoprotein). Besides, activated PBMCs with anti-CD3/CD28 Dynabeads (Gibco). After 24h (day 1), most of the T cells were activated.

2.4.3 Transduction of human primary T cells

On day 0, coat the non-tissue culture 6-well plates with 50 μg/mL Fibronectin dissolving in PBS (Biosharp), and stored overnight at 4. On day 1, moved the Fibronectin, blocking the wells for 0.5 hours with 2% bovine serum albumin (BSA) dissolving in PBS, then washing the wells for transduction. Added 200μL lentivirus encoding anti-BCMA CAR or MOCK per well, and centrifuged the 6-well plates at 1000xg for 2 hours at 32. Added 3×106 T cells to each well after centrifugation and centrifuged the 6-well plates at 300xg for 10 minutes at 32. Incubated the transduced T cells at 37degC with 5% CO_2 for subsequent use. During incubation, maintain the density of transduced T cells between 1-3x106 per mL in the T cell medium and replaced the new medium every two days. Transduced CAR T cells were used for flow cytometry analysis

on day 2 or 3, and used for in-vitro assays on day 3-7 post transduction. Moved anti-CD3/CD28 Dynabeads before use.

2.5 Cytokines, and ELISA assay

Pretreated Nalm-6 or Nalm-6-BCMA cells with or without BTZ (5nM) for 72 hours. Wash the tumor cells and the CAR T cells using blank RPMI-1640 media twice. Resuspend the tumor cells at 1x105 per mL, and the CAR T cells at 1-2x105 per mL. Tumor cells were cocultured with CAR T cells in 200μL blank RPMI-1640 media at effector to target ratio (E: T) 2:1 or 1:1 in 96-well plates in triplicates for 24h, collected cell-free supernatants for ELISA assay, and stored at -20 until use. Detecting cytokine (IFN-γ or TNF-α) secretion by ELISA kit (Biolegend).

2.6 Statistical analysis

All the data were expressed as the mean $\pm$ SD. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software). Statistical analysis of cytokine secretion was performed using paired Student's t-test. Statistical analysis of MFI was performed using multivariate analysis. P-values < 0.05 were considered statistically significant and P values are denoted with asterisks as follows (**** = p<0.0001, *** = p<0.001, ** = p<0.01, * = p<0.05, ns = p > 0.05).

3. Results

3.1 Bortezomib increased the expression of BCMA

Bortezomib showed a significant effect on the expression of some proteins, which undergo proteasomal degradation after ubiquitination has been confirmed by many studies. Recent studies revealed that the expression of claudin 1 protein increased by bortezomib in the human keratinocyte cell line HaCaT[6]. Bortezomib can also mitigate the negative effects of CD117 loss and CD28 abnormality, reported by Matevz Skerget et.al[7]. Therefore, we investigated whether or not the expression of BCMA is increased by bortezomib treatment in human MM cell line nalm6-BCMA. When we were doing a dosage test, the cell at 10nM bortezomib showed a significant decline within 72 hours, whereas that at 2.5nM and 5nM maintained a stable proliferation (Fig. 1) so we chose these two concentrations for further research. Flow cytometry using an anti-CD269 antibody showed that the expression of BCMA increased upon bortezomib treatment in a concentration-dependent manner (Fig.2 A and B). MFI analysis showed that the treatment with 5nM of BTZ resulted in a stable increase in BCMA expression. Compared with the 2.5nM group, the 5nM group had a more significant increase in fluorescence intensity, suggesting that 5nM would be an ideal concentration for CAR-T combination therapy (Fig.2 C). To examine whether the influence on BCMA was irreversible, flow cytometry was performed after the cells were washed twice with PBS and cultured for an extra 96 hours. We observed that BCMA increased in the presence of bortezomib, in addition, they were found to remain at a high level in the absence of BTZ. (Fig. 2D) The results showed that the proteasome inhibitor bortezomib increased BCMA steadily, resulting in promising lasting enhancement to CAR-T.

3.2 BTZ treatment of target cells effectively enhances CAR T-cell killing ability

To develop anti-BCMA CAR T-cell therapies against MM, we constructed a second-generation CAR-T targeting BCMA, including an alpaca heavy chain antibody VHH that specifically binds BCMA, fused to the CD8α-Dectin-CD3ζ intracellular signaling structural domain[8] (Fig. 3A). These CAR-T cells prepared by lentiviral transduction had high levels of CAR expression, and as a control, we used MOCK-transduced T cells with the same approach. In previous experiments, we have demonstrated that BTZ induces increased BCMA expression levels. We used 5nM BTZ-treated Nalm-6-BCMA cells as target cells based on the non-significant effect of BTZ treatment at this concentration on cell proliferation and the effective elevation of constitutive expression of the surface protein BCMA. After treating the cells for 72h in advance and confirming the increased expression of BCMA, we co-incubated the target cells with anti-BCMA CAR T for 24h according to different effector-to-target cell ratios (E: T) and analyzed the cytokine secretion in the supernatant using wild-type Nalm-6 cells and untreated Nalm-6-BCMA cells as controls (Fig. 3B). At 2:1 and 1:1 E: T, anti-BCMA CAR T cells produced significantly elevated secretion of cytokines IFN-γ and

TNF-α after co-cultured with 5nM BTZ-treated Nalm-6-BCMA cells, while MOCK T cells used as control caused lower values of cytokine secretion at any E: T ratio (Fig. 3C). The results suggested that MOCK T cells elicited low cytotoxicity and anti-BCMA CAR-T elicited high cytotoxicity, especially for Nalm-6-BCMA target cells with increased constitutive expression of the surface protein BCMA treated with BTZ. Figure 3 presented that the constructed anti-BCMA CAR-T induced a strong killing ability to targeted tumor cells in vitro, and increased killing ability compared to untreated group was closely related to the combined application of BTZ, which can be effectively and significantly enhanced after BTZ treatment.

Furthermore, the question of whether the combination of BTZ and anti-BCMA CAR-T cell therapy is effective in an in-vivo mouse model and the safety issues, as well as the timing and dosing of in-vivo treatment remain to be explored.

4. Discussion

At present, bortezomib combined chemotherapy and monoclonal antibody such as D-VTd[9] (daratumumab, thalidomide, and dexamethasone), R-CHOP[10] (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) are commonly used in the clinic. However, these drugs may cause various adverse effects, especially hematological toxicity, which was reported with VR-CAP (bortezomib, rituximab, cyclophosphamide, doxorubicin, and prednisone) and similar therapies in previous clinical trials[11]. Against this backdrop, we need to identify newer approaches for hematologic malignancies. Although there has been a large amount of research on multiple myeloma, few strategies like the combination of bortezomib with CAR-T therapy have been developed.

Recent studies offer inspiration to us that the BCMA, a valuable target in CAR-T therapy, is expressed uniformly on the malignant plasma cells of many patients with multiple myeloma[12]. The persistence of the upregulation of BCMA by BTZ provides more feasibility for the role of CAR T. Flow cytometry analyses showed that BCMA expression did not decrease in 96 hours after drug withdrawal. The retention time exceeded 72 hours, which was the time for the proteasome inhibition to return to the baseline[13]. Hence, it is reasonable to believe that the injection of CAR-T 72 hours after the treatment of BTZ into patients can be adopted, because this solution not only ensures the enhanced killing ability of CAR-T, but also avoids the damage to T cells caused by unmetabolized bortezomib.

In term of the mechanisms of BCMA up-regulation, we assumed that the bortezomib, as a proteasome inhibitor, can suppress the ubiquitination degradation of BCMA on the cell surface and thus enhance the expression. On the basis of this assumption, further research might shed light on the detailed mechanism. Moreover, the effect of bortezomib on BCMA upregulation in vivo is unknown. We suggest that the combination of CAR-T and bortezomib be investigated in more diverse cell lines and animal models. Overall, the current findings add substantially to our understanding of the CAR-T therapy of multiple myeloma, heralding the potential clinical value of the combination of bortezomib and CAR-T.

5.Conflict of Interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. Acknowledgements

7.Referances

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8.Legends

Fig.1 The proliferation of Nalm6-BCMA cell line treated with BTZ.

Fig.2 Increase in BCMA expression by BTZ. (A) Human MM cell line Nalm6-BCMA was treated with 2.5nM concentration for day 1, day 2, and day 3(D1, D2, D3). The green peaks are the untreated controls, which were cultured for the same amount of time as the experimental group. (B) Nalm6-BCMA cells were treated with 5nM BTZ for the indicated time, the orange peaks demonstrated a high level of BCMA expression. (C) Mean fluorescence intensity analysis. The flow results of the three time periods are summarized in group graphs. The MFI of the 2.5nM group and the 5nM group was compared before and after drug treatment. (D) Drug withdrawal test. The left picture shows increased expression under the action of 5nM BTZ, the right one shows the expression after drug withdrawal and culture for an extra 96h.

Fig 3. Increased cytotoxicity of anti-BCMA CAR T cells. (A) Anti-BCMA CAR is highly expressed on primary human T cells. (B) Sketch of BTZ-treated target cells co-cultured with anti-BCMA CAR T cells. (C) ELISA analysis of cytokine secretion in the supernatant after 24h of co-culture. p-values were calculated using a two-sided unpaired student's t-test (Mann–Whitney) or parametric student's t-test. All graphed data in this figure are represented as mean values ± SEM.

Data Availability

The data that support the findings of this study are available on request from the corresponding author, W.W,upon reasonable request.

A

A

B

C

D

A

| IL-2 SP | Anti-BCMA VHH | CD8α Hinge | Dectin TM+cyto | CD3 ζ |

B

C