Original Article Cyp1a1 is involved in drug resistance to bortezomib in CD138⁻ myeloma cells

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Abstract: One of the greatest challenges in multiple myeloma (MM) treatment is to overcome drug resistance. CD138⁻ MM cells show a more resistant phenotype to drugs than CD138⁺ cells. In this study, we demonstrated that CD138⁻ cells are more resistant to bortezomib than CD138⁺ cells, mainly due to the higher cyp1a1 activity in CD138⁻ cells. Suppressing cyp1a1 activity in CD138⁻ cells by Notch inhibition, NF (cyp1a1 inhibitor) or cyp1a1 siRNA results in increased sensitivity of CD138⁻ cells to bortezomib treatment. Up-regulation of cpy1a1 activity in CD138⁺ cells by Dll1 simulation induced drug resistance to bortezomib. Our data showed that cyp1a1 activity is involved in drug resistance to bortezomib in CD138⁻ MM cells. It provided a new strategy of suppressing cyp1a1 activity to overcome drug resistance to bortezomib in clinical treatment.

Keywords: Myeloma, drug resistance, CD138, bortezomib, cyp1a1

Introduction

Multiple myeloma (MM) is an incurable plasma cell malignancy characterized by clonal proliferation of plasma cells and secretion of monoclonal immunoglobulin. Patients with MM are at an increased risk of developing infections, anemia, thrombocytopenia, renal failure, and bone disease [1, 2]. The introduction of novel agents, including bortezomib in combination with autologous stem cell transplantation, has led to a significant advancement in the treatment of MM patients [3-6]. However, drug resistance is frequently induced during chemotherapy and become one of the major issues in MM clinical treatment. The bone marrow microenvironment is important in MM cell growth and survival and also contributes to drug resistance in MM [7, 8]. Previously we demonstrated that Notch activation by bone marrow stromal cells could induce drug resistance to bortezomib by up-regulating Cyp1a1 expression [9]. Cyp1a1 enzyme could accelerate bortezomib drug metabolism thus reducing the drug concentration in the cells [9]. Several studies reported that CD138⁺ and CD138⁻ cells have different properties in cell proliferation, clonogenic growth, tumor initiation ability and drug resistance [10-14]. In this study, we analyzed the different response of CD138⁺ and CD138⁻ myeloma cells in drug sensitivity to bortezomib and investigated the molecular mechanism. We demonstrated that CD138⁻ cells have a higher Notch activation level with higher cyp1a1 expression and activity, which contribute to bortezomib resistance of CD138⁻ cells compared to CD138⁺ MM cells.

Material and methods

Cell culture

We used the LP-1 multiple myeloma cell line [15] in this study. These suspension cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 50 μ g/mL streptomycin (Corning, Ithaca, NY, USA). Cells were cultured at 37°C in an incubator (Thermo Scientific Varioskan Flash, Waltham, MA, USA) containing 5% CO₂. Cells were refreshed 24 h before performing experiments.

FACS sorting for CD138⁺ and CD138⁻ cells

Lp-1 cells were stained with CD138⁻ PE at 4°C for 30 mins; we gated the 30% of the most positive cells as CD138⁺ cells and selected 30% of the most negative cells as CD138⁻ cells by flow cytometry (BD, Franklin Lakes, NJ, USA) for further experiments. The sorted cells were checked for purity by flow cytometry and the purity for each group was higher than 90%.

Cell viability assay

A CellTiter-Glo assay (Promega, Madison, WI, USA) was used to assess cell viability based on the quantification of ATP [16]. Drug sensitivity to bortezomib was detected by incubation with 5 nM (final concentration) bortezomib in the cells. 24 h after bortezomib treatment, 100 μ L of cell suspension were added to 100 μ L of luminometric reagent in a 96-well nontransparent plate. After incubation at room temperature for 10 min, the luminescence intensity was recorded using the microplate reader (Thermo) using the protocol for luminometric measurement.

Real-time PCR

Cells were lysed in 250 µL Trizol (Invitrogen, Carlsbad, USA) and total RNA was extracted using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). RNA was converted into cDNA by the SuperScript First-Strand Synthesis System (Invitrogen) using random hexamers as primers. Real-time PCR were performed on Bio-Rad CFX Connect™ Real-time System (Bio-Rad, Foster City, CA, USA) Primer sequences were described in our previous studies.

Cyp1a1 enzyme activity assay

MM cells were plated in 96-well optical plates at a concentration of 10⁵ cells/well and Cyp1a1 enzyme activity is measured by P450-Glo[™] Cyp1a1 Assay (#v8751) from Promega according to the manufacturer's instructions. Each experimental condition was performed at least 3 times.

Transient transfection of cyp1a1 siRNA

LP-1 cells were transient transfected with cyp-1a1 siRNA(h) (sc-41483) and control siRNA (sc-37007) (Santa Cruz Biotechnology, CA, USA) by using the siRNA Reagent System (sc-45064) (Santa Cruz Biotechnology) according to the manufacturer's instructions. Details were described in our previous study [9]. For FITC conjugated control siRNA, it was used to detect transfection efficiency by flow cytometry.

Annexin-V and PI staining

The apoptosis of LP-1 cells after bortezomib treatment was detected by flow cytometry using an Annexin-V/PI apoptosis kit (BD). Cells were harvested and washed twice with DPBS without calcium and magnesium (Corning). Cells were resuspended in 50 μ L 1× binding buffer with 2 μ L annexinV-APC (3 μ g/mL) and 2 μ L PI (50 μ g/mL) and incubated at room temperature in the dark for 15 min. An additional 400 μ L 1× binding buffer was added, and samples were analyzed by flow cytometry.

Western blot

Cell pellets were lysed in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Nonidet P40, and 0.25% sodium deoxycholate. Cell debris was removed by centrifugation for 5 min at 4000× g before sample buffer was added. After boiling, samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Bio-Rad), which were blocked with PBS containing 5% low-fat milk and 0.1% Tween 20. Membranes were probed with antibodies against human cyp1a1 (1:500) and β-actin (1:2000) (Cell Signaling Technology, Danvers, MA, USA). Membranes were washed with PBS containing 0.1% Tween 20 (PBST) for 30 min and then incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2000) for 30 min at room temperature. Membranes were washed in PBST and imaged using a ChemiDoc-It 510 system (UVP, Upland, CA, USA).

Notch simulation by recombined human DII1 ligand

Recombinant humanDll1 (R&D Systems, Minneapolis, USA) was dissolved in phosphate-buffered saline (PBS) and immobilized in 96-well plates for 20 hours at 4°C, at 0.5 μ g/mL. As control for Dll1, control IgG (R&D Systems) was used. Plates coated with Dll1 or IgG were washed with PBS and 10⁵ LP-1 cells were plated for simulation.



Figure 1. Different properties of CD138⁺ and CD138⁻ MM cells in bortezomib resistance, Notch activation and cyp1a1 activity. A: Purity of CD138⁺ and CD138⁻ cells after FACS sorting of LP-1 MM cells. B: Drug sensitivity to bortezomib of CD138⁺ and CD138⁻ cells after 24 h of bortezomib treatment. C: Different expression of Notch target genes in CD138⁺ and CD138⁻ cells. D: Cyp1a1 mRNA expression in CD138⁺ and CD138⁻ cells. E: Cyp1a1 enzyme activity in CD138⁺ and CD138⁻ cells.

Statistical analysis

All experiments were repeated at least 3 times. Data were presented as mean \pm SD (standard deviation). The significance between groups was determined using the Mann-Whitney U test. The results were considered significant when P<0.05.

Results

Different properties of CD138⁺ and CD138⁻ MM cells in bortezomib resistance, Notch activation and cyp1a1 activity

To investigate the different property of CD138⁺ and CD138⁻ cells in LP-1 MM cells, we used FACS sorting to collect CD138^{+/-} sub-populations. 30% of the most positive cells were col-

lected as CD138⁺ cells and 30% of the most negative cells were sorted as CD138⁻ cells. We first checked the purity after FACS sorting and it showed that the purity was higher than 90% (Figure 1A). We then analyzed the drug sensitivity to bortezomib in both sub-populations and it showed that CD138⁻ cells were more resistant to bortezomib treatment for 24 h (Figure 1B). Previously, we demonstrated that cyp1a1 up-regulation by Notch activation contributed to bortezomib in myeloma cells. So we further analyzed the expression of Notch target genes in CD138⁻ and CD138⁺ cells. Real-time PCR results showed that the Notch activation level was higher in CD138⁻ cells compared to CD138⁺ cells (Figure 1C). Meanwhile, cyp1a1 mRNA expression (Figure 1D) and cyp1a1 enzyme activity (Figure 1E) were much higher in CD138⁻ cells than that in CD138⁺ cells.



Figure 2. Inhibiting Notch by DAPT increases drug sensitivity to bortezomib in CD138⁻ cells. A: Cyp1a1 mRNA expression in CD138⁻ cells after DAPT treatment for 48 h. B: Cyp1a1 enzyme activity in CD138⁻ cells after DAPT treatment for 48 h. C: Drug sensitivity to bortezomib after DAPT treatment of CD138⁻ cells for 48 h.



Figure 3. Suppressing cyp1a1 activity by NF increases sensitivity to bortezomib. A: Cyp1a1 mRNA expression after different concentration of NF treatment for 24 h in CD138⁻ cells. B: Cyp1a1 enzyme activity after different concentration of NF treatment for 24 h in CD138⁻ cells. C: Drug sensitivity to bortezomib after NF treatment for 24 h in CD138⁻ cells.

Inhibiting Notch by DAPT increases drug sensitivity to bortezomib in CD138⁻ cells

Next, we used DAPT, a Notch pathway inhibitor, to down-regulate Notch signaling in CD138⁻ cells, and investigate cyp1a1 expression and drug sensitivity to bortezomib. Real-time PCR showed that the expression of cyp1a1 was decreased after DAPT treatment (final concentration of 10 uM) for 48 h (Figure 2A). The enzyme activity was also decreased by blocking Notch pathway for 48 h (Figure 2B). Moreover, Notch inhibiting by DAPT in CD138⁻ cells resulted in increased sensitivity to bortezomib treatment for 48 h (Figure 2C), indicating that cyp1a1 may be involved in drug resistance of bortezomib in CD138⁻ cells.

Suppressing cyp1a1 activity by NF increases sensitivity to bortezomib in CD138⁻ cells

To confirm the role of cyp1a1 in drug resistance to bortezomib in CD138⁻ cells. A cyp1a1 enzyme inhibitor, NF (α -Naphthoflavone) was used in the experiment. We treated CD138⁻ cells in two final concentration of NF (20 uM and 40 uM) for 24 h and found that it has little effect on MM cell viability (data not shown) or cyp1a1 mRNA expression (**Figure 3A**). However, NF could reduce cyp1a1 enzyme activity in CD138⁻ cells in 24 h (**Figure 3B**). Furthermore, inhibiting cyp1a1 activity by NF for 24 h, CD138⁻ cells became sensitive to bortezomib treatment and resulted in less cell viability (**Figure 3C**).



Figure 4. Cyp1a1 siRNA increases drug sensitivity to bortezomib in CD138 cells. A: siRNA transfection efficiency detected by flow cytometry. B, C: mRNA and protein expression of cyp1a1 after siRNA transfection for 48 h in CD138 cells. D: Cyp1a1 enzyme activity after siRNA transfection for 48 h in CD138 cells. E: Drug sensitivity to bortezomib detected by viability assay after siRNA transfection for 48 h in CD138 cells. F: Drug sensitivity to bortezomib detected by apoptosis assay after siRNA transfection for 48 h in CD138 cells.

Cyp1a1 siRNA increases drug sensitivity to bortezomib in CD138⁻ cells

Next, cyp1a1 siRNA was used to down-regulate cyp1a1 expression in CD138⁻ cells. A scramble siRNA was used as control. We tested the transfection efficiency by flow cytometry and it showed the efficiency was more than 90% (**Figure 4A**). By real-time PCR we showed that

cyp1a1 mRNA expression was decreased after transfection with cyp1a1 siRNA for 48 h compared to control (**Figure 4B**). The protein expression was also decreased by siRNA knocking down as measured by western blot (**Figure 4C**). Furthermore, enzyme activity assay showed that cyp1a1 siRNA could suppress cyp1a1 activity in CD138⁻ cells (**Figure 4D**). Cell viability assay indicated that down-regulation of cyp1a1



Figure 5. Up-regulation of cyp1a1 in CD138⁺ cells by Dll1 simulation reduced the sensitivity to bortezomib treatment. A: Expression of Notch target genes after Dll1 simulation for 48 h in CD138⁺ cells. B, C: mRNA and protein expression of cyp1a1 after Dll1 simulation for 48 h in CD138⁺ cells. D: Cyp1a1 enzyme activity after Dll1 simulation for 48 h in CD138⁺ cells. E: Drug sensitivity to bortezomib after Dll1 simulation for 48 h in CD138⁺ cells.

expression by siRNA for 48 h could increase the sensitivity to bortezomib treatment (**Figure 4E**), resulting in an increasing apoptotic MM cells detected by AnnexinV/PI staining after bortezomib treatment for 24 h (**Figure 4F**).

Up-regulation of cyp1a1 in CD138⁺ cells by Dll1 simulation reduced the sensitivity to bortezomib

Since CD138⁺ cells are more sensitive to bortezomib than CD138⁻ cells. We tried to up-regulate cyp1a1 expression in CD138⁺ cells by Notch activation. Dll1 ligand simulation of CD138⁺ cells for 48 h could activate Notch pathway as the downstream target genes were up-regulated as measured by real-time PCR (**Figure 5A**). Cyp1a1 expression was up-regulated in CD138⁺ cells by Dll1 simulation for 48 h both in mRNA level (**Figure 5B**) and in protein level (**Figure 5C**). Cyp1a1 enzyme activity was increased by Dll1 simulation for 48 h in CD138⁺ cells (**Figure 5D**), resulting in less drug sensitivity to bortezomib treatment for 24 h as detected by viability assay (**Figure 5E**).

Discussion

Drug resistance is one of the major problems encountered in clinical therapy. Bortezomib is a first line drug used in the standard treatment and has improved clinical outcome. Previous studies demonstrated that CD138 and CD138⁺ cell subpopulations showed distinguish properties and different response to drug resistance, yet the mechanism of drug resistance has not been well described. In our previous study, we reported that Notch activation could induce drug resistance to bortezomib in MM cells by up-regulating cyp1a1. Cyp1a1 is one of the main cytochrome P450 enzymes that play a pivotal role in the metabo-

lism of a wide variety of xenobiotic and endogenous compounds [17, 18]. Cyp1a1 shows similar function to cyp1a2, an enzyme that was involved in metabolism of bortezomib [19-21]. Interestingly, CD138⁻ cells showed a much high level of Notch activation than CD138⁺ cells. Meanwhile, CD138⁻ cells have a higher expression of cyp1a1 and a higher cyp1a1 enzyme activity. We suppressed the cyp1a1 enzyme activity in CD138⁻ cells by Notch inhibition or cyp1a1 inhibitor and found that CD138⁻ cells become sensitive to bortezomib treatment. Using cyp1a1 siRNA to down-regulate cyp1a1 mRNA expression could also increase the sensitivity to bortezomib, indicating that cyp1a1 is involved in drug resistance in CD138⁻ subpopulation. To further confirm this idea, we upregulated cyp1a1 in CD138⁺ cells by Notch activation, and found that CD138⁺ cells turned to be more resistance to bortezomib. Our data reported for the first time that cyp1a1 is an important factor in drug resistance of bortezomib in CD138⁻ cells. In myeloma treatment, CD138⁺ cells are more sensitive to chemotherapy [22, 23], CD138⁻ cells are the key target to overcome the drug resistance in clinical therapy. The combination of cyp1a1 inhibitor with bortezomib as a potential therapy shows a promising prospects to increase drug sensitivity and prolong the clinical outcomes.

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Disclosure of conflict of interest

None.

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