Bortezomib as a new therapeutic approach for blastic plasmacytoid dendritic cell neoplasm

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Online supplement

Cell lines and culture

Data from patients #24, 86, 66 and 38 have been previously published [1, 2]. Cells were cultured at 10^6 cell/ml in RPMI-1640 glutamax medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 1% penicillin/streptomycin (PAA Laboratoires, Vélizy-Villacoublay, France) with or without chemotherapeutic drugs at 37°C under 5% CO2 for 24 or 48 hours. We also developed a luciferase-expressing BPDCN cell line (Luc$^+$ CAL-1 cell line) in order to monitoring tumor burden *in vivo* mouse model without mouse sacrifice.

Drug and culture

Bortezomib (20 nM) were tested in association with cytotoxic drugs against BPDCN cells: idarubicin (I) at 0.03 µM, dexamethasone (D) at 0.637 mM, obtained from the Pharmacy of the University Hospital of Besançon (Pr Limat). Bortezomib (20 nM) were also tested in association with HDAC (histone deacetylase) inhibitor like vorinostat (SAHA) at 1.25 µM, 5-azacytidine (5-Aza) at 4µM, statin such as pravastatin (P) and simvastatin (S) at 5 µM from Sigma Aldrich, Saint-Quentin Fallavier, France. The SL-401 drug, a novel targeted therapy directed to IL-3R (Stemline Therapeutics, New York, NY, USA) was used at 365 pM, as positive control, since BPDCN cells are sensitive to SL-401 [2].

Cytotoxicity, proliferation and cell cycle assay by flow cytometry

The following monoclonal antibodies were used to gate BPDCN cells: Horizon V500 (V500)–conjugated CD45 (HI30, BD Biosciences, Le Pont de Claix, France), Horizon V450 (V450)-CD56 (B156, BD Biosciences), phycoerythrin-cyanin-7 (PE-Cy7)-CD123 (6H6, Biolegend, Ozyme, Saint-Quentin en Yvelines, France), phycoerythrin (PE)-BDCA-4 (12C2, Biolegend) and allophycocyanin-H7 (APC-H7)-CD4 (SK3, BD Biosciences). Flow cytometry

was performed on a FACSCANTO II cytometer (BD Biosciences, Le Pont de Claix, France) using DIVA 6.3 software for acquisition and analysis (BD Biosciences). For cell cycle assay, cells were fixed with 70% ethanol and incubated overnight at +4°C. Then, the cells were stained with 300 µl Phosphate buffer saline (PBS) (Gibco, Life Technologies, Saint-Aubin, France), 5 µl propidium iodide (Sigma Aldrich, Saint Quentin Fallavier, France) and 1 µl RNase (Sigma Aldrich) before cytometry acquisition (FC500, Beckman Coulter).

NF-κB pathway activation

CAL-1 cells (10^6 cells/ml) were incubated with bortezomib from 25 to 75 nM for 6 hours. After fixation and permeabilization, NF-κB pathway stimulation was investigated through phosphorylated-NF-κB subunit RelA (pRelA) staining, using Alexa fluor 647 conjugated anti-NF-κB p65 (K10-895.12.50, BD Biosciences) antibody or isotype control (IgG2b kappa, MOPC 173, BD Biosciences) for experiments performed *in vitro.* An APC anti-NF-κB p65 (REA 348, Miltenyi Biotec, Bergisch Gladbach, Germany) antibody or its isotype control (REA control isotype APC) were used in PDX cells extracted from mouse blood after *in vivo* treatment [3]. In CAL-1 cells, nuclear and cytoplasmic pRelA expression was also evaluated by confocal microscopy using FV1000 confocal microscope (Olympus, Rungis, France) and analyzed with Olympus FV-viewer software, as described [3]. Cells were washed and immobilized into glass microscope slides by cytospin's centrifugation (1400 rpm, 5 min), before fixation and permeabilization using methanol (20 minutes at -20°C). Then, cells were washed 3 times with PBS 0,5% FBS solution, before staining with the primary antibody (anti-NF-κB p65 pS536, clone J144-460, BD Bioscience) followed by overnight incubation at 4°C with the secondary antibody (Alexa fluor 555-conjugated goat anti-mouse IgG (H+L), Life technologies, Gaithersburg, Maryland), for 1 hour at room temperature. Cell nuclei were

stained with DAPI (Sigma Aldrich), and finally, cells were washed and mounted (Fluorescent Mounting Medium, Dako, Trappes, France) before acquisition.

Primary BPDCN cell xenograft model

Mice were then randomized and allocated to the two treatment groups namely: bortezomib (0.25 mg/kg, intraperitoneally) once (n=7) or twice (n=4) a week during 4 weeks, or PBS (control, n=3) in 2 independent experiments. Engraftment was confirmed by the identification of human primary leukemic cells in mouse peripheral blood using cytometry. Circulating human leukemic cells were weekly investigated in mice blood by cytometry, using APC-anti-mouse CD45 antibody (30F11, BD Biosciences), as well as V500-anti-human CD45, PE-CY7-CD123, V450-CD56, PerCP-cy5.5-CD3, APC-H7-CD4, and PE-CD304 (AD5-17F6, Miltenyi Biotec) to identify Human BPDCN cells. Cell counting was performed using TruCount® beads (BD Biosciences) according to manufacturer's instructions. In other experiments, NSG mice were then randomized and allocated to one of the three treatment groups namely: bortezomib (0.25 mg/kg, intraperitoneally), JSH23 (40 mg/kg) used as positive control, or PBS (control) used as negative control. Mice were examined daily for overall activity and for the presence of a tumoral syndrome, and they were sacrificed whenever appropriate. These procedures were carried out in accordance with the guidelines for animal experimentation and according to a protocol approved by Veterinary Services for Animal Health & Protection, issued by the Ministry for Agriculture, Paris, France (protocol 11007R).

Luciferase CAL-1 cell assay and *in vivo* bioluminescence imaging

The CAL-1 cell-line was transduced with a Luc-retroviral vector carrying Luciferase (Luc^{+}) and neomycin resistance (NeoR) genes. NOD/Shi-SCID/IL-2Rγc^{null} (NOG mice, 6-8

weeks of age, CIEA, Japan) were injected intravenously with 0.5; 1 or 5.10^6 of Luc^+ CAL-1 cells (n=3/group). Transduced cells were then cloned at 0.3 cells/well. Two luciferase-expressing (Luc^+) cell clones were harvested according to their luminescence intensity emission measured using the NightOwl LB983 imaging system and analyzed with the WinLightTM software 5 (Berthold technologies, Germany).

NOD/Shi-SCID/IL-2Rγcnull (NOG mice, 6-8 weeks of age, CIEA, Japan, total n=9 mice) were injected intravenously with 0.5; 1 or 5.106 of Luc^+ CAL-1, (n=3/group). E-luciferin (Promega, Lyon, France) was injected into the mouse peritoneal cavity (150 mg/kg) 10 minutes prior imaging. Circulating Luc^+ CAL-1 cells were monitored by flow cytometry. Monitoring of Luc^+ CAL-1 cell tumor burden was performed at day 6, 8, 12, and 15 by measuring luciferase activity after luciferin infusion by total body bioluminescence imaging (BLI, NightOwl). BLI and WinLight analysis (photon flux) allowed us to evaluate CAL-1 cell tumor burden at day 6, 8, 12, and 15. At the end of the experiment, mice were euthanized and different organs (the lungs, bone marrow, the spleen, the liver, kidneys, the pancreas, spinal cord, ovary, and lymph nodes) were harvested and imaged by BLI. Leukemic cell morphology was performed on cytospins from murine blood or spleen samples after May Grunwald Giemsa staining (Sigma-Aldrich), as described [4].

Statistical analysis

Data were presented as the mean ± Standard Error of the Mean (SEM). A *p* value <0.05 was considered statistically significant.

Table 1. Characteristics of the 7 BPDCN patients who provide primary BPDCN cells.

Patient number	Age/ gender	Cutaneous lesions	Sites of disease involvement	Phenotype	Karyotype
#24	55/M	yes	LN/BM	$CD4^{+}$ $CD56^{+}$ $CD123^{+}$ $CD38^{+}$	NA
#25	75/M	no	LN/BM	$CD4^{+}$ $CD56^{+}$ $CD123^{++}$ $CD303^{+}$ $CD304^{+}$ My^{-} B^{-} $CD7^{+}$ (others T^{-})	45,XY,-5[3],46XY[10]
#69	57/M	no	BM	$CD4^{+}$ CD123 $CD303^{+}$ $CD304^{+}$ $CD7^{+}$ $CD33^{+}$	46XY,der'3)?c,t(7;46;10)(p15;q2?4;q2?3)[4]/47,,t8[3]/47,sdl1,der(20)(?)[2]/46, XY, der(3)?c[6] t(7;16;10), trisomiy 8
#86	63/M	yes	LN/ Splenomegaly/BM	$CD4^{+}$ $CD56^{+}$ $CD123^{++}$ $CD303^{+}$ $CD304^{+}$ $TCL1^{+}$ My^{-} B^{-} T^{-}	44,X,-Y,-13[3]/44,sl,i(7)(q10)[15]/45,XY,-15[3]
#127	90/M	NA	NA	$CD4^{+}$ $CD56^{+}$ $CD123^{++}$ $CD303^{+}$ $CD304^{+}$ $TCL1^{+}$ My^{-} B^{-} T^{-}	NA
#66	70/F	yes	skin	$CD4^{+}$ $CD56^{+}$ $CD123^{++}$ $CD303^{+}$ $CD304^{+}$ $TCL1^{+}$ My^{-} B^{-} T^{-}	46,XX i(7q)
#38	81/M	yes	LN/ splenomegaly/BM	$CD4^{+}$ $CD56^{+}$ $CD123^{++}$ $CD303^{-}$ $CD304^{+}$ $TCL1^{+}$ My^{-} B^{-} $CD7^{+}$ (others T^{-})	43-44, XY,der(2) t(2;?)(q23;?),der(4)t(4;?)(q35;?),5,der(7)t(7;?)(q31;?),-9,-10,-13,-15,-16,del(17)(p11),+1-4mar[17];8283,idemx2 [12];46,XY

Age (years)/gender; Results of phenotypic analysis performed on blood or bone marrow samples (flow cytometry); karyotype; presence of cutaneous lesions; histopathological diagnosis. My: myeloid markers (including myeloperoxidase, CD13, CD33, CD117, CD15, CD65, CD14, CD64); T : T lymphoid markers (including membrane CD3, intracytoplasmic CD3, CD7, CD5, CD2, CD8); B: B lymphoid markers (including intracytoplasmic CD79a, intracytoplasmic CD22, intracytoplasmic Ig µ chains, CD19, CD20, CD22, surface immunoglobulin); +: positive expression; ++: high expression; -: no expression; NA: not available; LN: lymph nodes; BM: bone marrow.

Supplemental references

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