The cytotoxicity of anti-CD22 immunotoxin is enhanced by bryostatin 1 in B-cell lymphomas through CD22 upregulation and PKC-βII depletion

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Supplementary Design and Methods

Separation procedures

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by centrifugation over a Ficoll-Hypaque layer (Biochrom, Berlin, Germany) of 1.077 g/mL density. For separation of CLL B cells, PBMC were incubated with anti-CD2 and anti-CD14 magnetic beads (Dynabeads M450, Dynal, Oslo, Norway) according to the manufacturer's instructions. After separation, B cells from CLL patients were over 98% pure as assessed by direct immunofluorescence. Cells were harvested under ice-cold conditions and were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum at 37°C and 5% CO_2 in a fully humidified atmosphere.

Culture conditions

Purified B cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (PAA, Pasching, Austria), penicillin/streptomycin 50 IU/mL, Na-pyruvate 1 mM, L-glutamine 2mM, L-asparagine 20 μg/mL, 2-mercaptoethanol 0.05 mM, HEPES 10 mM and MEM non-essential aminoacids 0.7x (Invitrogen) at 37°C and 5% CO_2 in a fully humidified atmosphere.

For imitating feeder cell support, purified leukemic B lymphocytes were cultured in the presence of the murine fibroblast cell line Ltk^-.[1] (provided by Paloma Perez Aciego, Madrid, Spain) in the medium indicated above. CLL cells were cultured in 12-well flat-bottom plates with $2x10^5$ Ltk^- cells per well. To inhibit further progeny of feeder cells during co-culturing, Ltk^- cells were pre-treated with mitomycin C (provided by Sigma) 10 μg/mL for 2 h and afterwards washed 3 times with PBS before starting co-culture.

Reagents and antibodies

Bryostatin 1 was obtained from Biomol, Hamburg, Germany. PD98059 was purchased from Calbiochem/ Merck, Darmstadt, Germany. The following antibodies were used for immunoblotting as indicated in the experiments: Mcl-1, PKC-ßII (clone C-18), (Santa Cruz Biotechnology, Santa Cruz, CA, USA). XIAP, Bcl-2, PKC ε (BD Biosciences, San Jose, CA, USA), phospho-Bcl-2 (serine70), β-actin (Sigma-Aldrich, St. Louis, MO, USA). Noxa and Puma-antibodies were obtained from ProSci, Poway, CA, USA. Enzastaurin was kindly provided by the Lilly Corporation.

Analysis of apoptosis

Cells to be examined for annexin-V expression were washed with PBS and re-suspended with 500 μL binding buffer (Annexin-V-FITC kit, Immunotec, Marseille, France) containing 1 μL Annexin-V-fluorescin-isothiocyanate stock and 5 μL 20 μg/mL phosphatidyl-inositol (PI) to determine the phosphatidylserine (PS) exposure on the outer plasma membrane. After incubation for 10 min at room temperature in a light protected area the specimens were quantified by flow cytometry.

Immunophenotyping

Cells were washed in PBC containing 2% FCS and incubated with saturating amounts of fluorochrome-conjugated monoclonal antibody or an equal amount of non-binding, isotype-identical control antibody. Fluorochromes were either FITC- or PE. After 30 min at 4°C, cells were repeatedly washed with PBS/2% FCS and analyzed via flow-cytometry using a Coulter Epics XL cytofluorometer, acquiring 10,000 events. Data were analyzed using WinMDI 2.8 FACS software. The relative expression of surface antigen is described as the mean fluorescence intensity ratio. This equals the mean fluorescence intensity of cells stained with a fluorochrome-conjugated antigen-specific antibody, divided by the mean fluorescence intensity of cells stained with a fluorochrome-conjugated isotype control antibody.

Immunoblotting

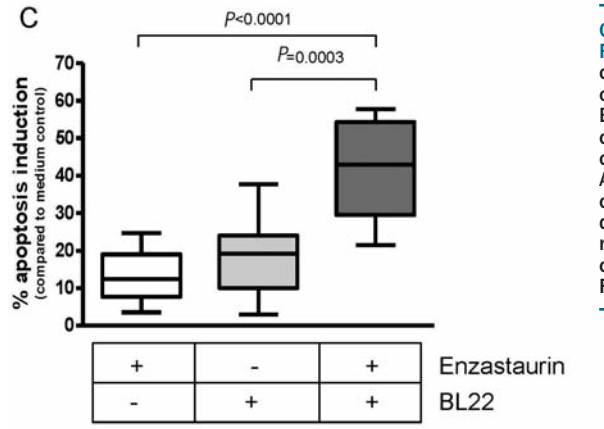
To investigate cell proteins via Western blot, $1\text{-}10x10^7$ cells were lysed in lysis buffer (10 μM Tris / HCl (ph 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 10 mg/mL of each phenantroline, aprotinine, leupeptine and pepstatine) for 20 min at 4°C. Lysates were spun at 12,000 rpm for 20 min and supernatants were collected. Protein concentration was assessed by the Bio-Rad assay method (Bio-Rad Laboratories, Hercules, CA, USA). Total extracts (40 μg/lane) were subjected to SDS-Page and blotting was performed on PVDF membranes (Immobilon-P, Millipore GmbH, Germany).

Transfection of CD22 siRNA

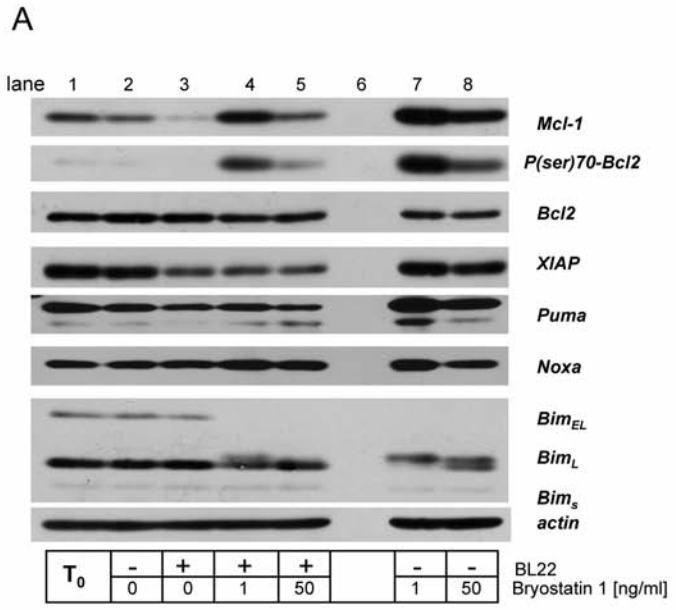
For siRNA-mediated downregulation of CD22 an ON-TARGETplus SMARTpool siRNA against human CD22 was used, purchased from Thermo Scientific (Lafayette, CO, USA). Non-binding siRNA was used as control, and Alexa Fluor 488 conjugated non-binding siRNA, (Qiagen, Hilden, Germany) served to evaluate transfection efficiency. Typically, $1x10^7$ cells were re-suspended in 100 μL of Cell Line Nucleofector solution V and mixed with 3 μg of siRNA. Nucleofections were performed using the X-001 program of the Amaxa Nucleofector II device (Amaxa Biosystems GmbH, Cologne, Germany). Immediately after transfection, cells were transferred into RPMI containing 10% fetal bovine serum.

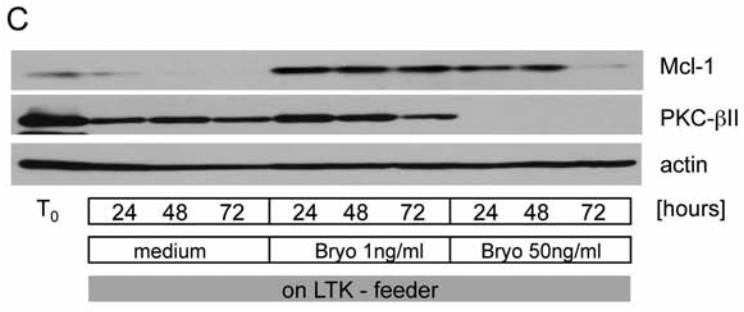
References

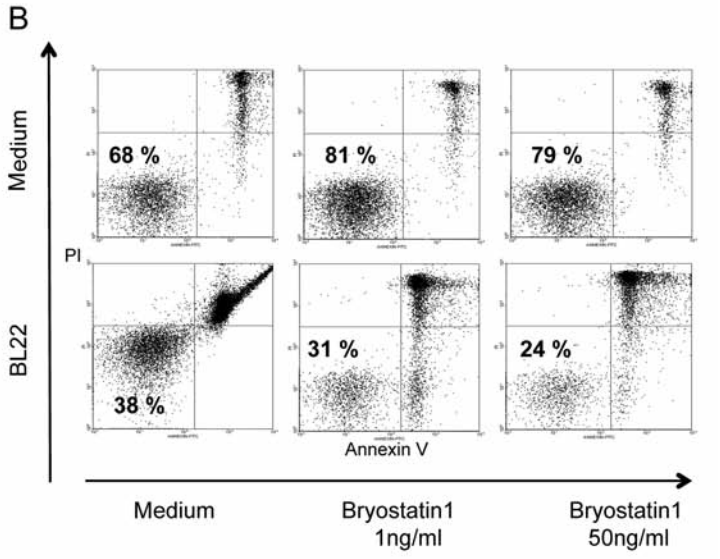
1. Cuni S, Perez-Aciego P, Perez-Chacon G, Vargas JA, Sanchez A, Martin-Saavedra FM, et al. A sustained activation of PI3K/NF-kappaB pathway is critical for the survival of chronic lymphocytic leukemia B cells. Leukemia. 2004;18(8):1391-400.



Online Supplementary Figure S1. CLL cells were cultured in the presence of enzastaurin (5μM), BL22 (1ng/mL) or a combination of both compounds for 48 h. Apoptosis-induction was calculated based on the difference to spontaneous apoptosis in cells cultured in RPMI/10% FCS alone (n=11).







Online Supplementary Figure S2. BL22 overcomes bryostatin 1-dependent upregulation of Mcl-1 and phospho-serine70-Bcl-2. (A) CLL cells were stimulated with bryostatin 1 in the absence or presence of BL22 (1 μg/mL). After 24 h cells were analyzed for the expression of anti- and pro-apoptotic proteins. T$_0$ indicates cell lysates from CLL cells freshly isolated from peripheral blood. Two additional experiments revealed identical results. (B) Single agent treatment with bryostatin 1 decreases spontaneous cell death (upper row): CLL cells were cultured in medium supplemented with bryostatin 1 for 24 h. Viable cells were quantified based on negativity for Annexin-V and PI based on flow cytometry analysis. The combination of BL22 and bryostatin 1 increases cytotoxicity of the immunotoxin in spite of up-regulated Mcl-1 and phosphorylation of Bcl-2 (lower row). (C) Regulation of Mcl-1 and PKC-βII by bryostatin 1 on stromal cells. Primary CLL cells were co-cultured for 72 h on Ltk$^-$ cells. After the time points as indicated, CLL cells were removed from the co-culture and the expression of Mcl-1 and PKC-βII assessed by immunoblotting. Similar to the experiments performed in RPMI/FCS, bryostatin 1 up-regulated Mcl-1 and down-regulated PKC-βII in a dose-dependent manner.

Online Supplementary Table S1. Patients' characteristics.

ID	Age and sex	Binet stage	CD38 expression	ZAP70 expression	IgH mutation	Previous therapies	% apoptosis induction BL22	% apoptosis induction BL22+Bryo1	% apoptosis induction BL22+Bryo50
V125	73m	B	positive	negative	n.d.	0	-3.8	12.1	33
V132	83m	A	negative	negative	n.d.	Chlorambucil	4.1	17.9	59
V180	44m	A	negative	negative	mutated	0	13.2	17.9	34.4
V190	70f	A	negative	negative	n.d.	0	7.5	17.4	48.8
V194	67m	A	negative	positive	n.d.	0	0.9	64.2	78.5
V204	55m	C	negative	positive	unmutated	R-FC, R-CHOP, R-Bendamustine	2.7	0.4	0
V225	45f	B	partial	positive	unmutated	Fludarabine, Dexa-BEAM, TBI, ASCT	4.4	42.8	69.5
V226	84f	C	partial	positive	n.d.	Chlorambucil	33.0	34.6	45.7
V231	68f	C	negative	positive	n.d.	0	2.1	10.6	29.7
P8	80m	B	negative	negative	n.d.	0	17.8	21.8	24.1
P16	77m	C	n.d	positive	unmutated	Chlorambucil	15.7	57.2	71.9
V3	78f	A	negative	n.d.	n.d.	0	10.0	34.2	48.5

*n.d.: not done; * ZAP70 positivity in case >20% of CLL cells were positive by flow cytometry analysis.*