SUPPLEMENTARY DATA

SUPPLEMENTARY METHODS

Isolation and culture of primary cells

Peripheral blood mononuclear cells or bone marrow aspirates from CLL patients were isolated by Ficoll-paque sedimentation (GE-healthcare). Tumor cells from lymph nodes were obtained after squirting with RPMI 1640 (Life technologies) culture medium using a fine needle. Cells were either used directly or cryopreserved in liquid nitrogen in RPMI 1640 containing 10% DMSO (Sigma) and 60% heat-inactivated fetal bovine serum (FBS; Life technologies). Manipulation due to freezing/thawing did not influence cell response. After thawing, cells were cultured in RPMI 1640, supplemented with 10% FBS, 2mM glutamine (Life technologies) and 50μg/mL penicillin-streptomycin (Life technologies), in a humidified atmosphere at 37ºC containing 5% carbon dioxide.

Antibodies

Membranes were probed with the following primary antibodies: phospho-mTOR (Ser2448), mTOR, phospho-Akt (Ser473), phospho-S6 ribosomal protein (S6RP; Ser235/236), S6RP (clone 54D2), phospho-4E-BP1 (Thr37/46), 4E-BP1 (clone 53H11), phospho-eIF4E (Ser209), eIF4E, phospho-FoxO3a (Thr32) (clone 4G6) and Akt from Cell Signaling Technology, FoxO3a (Upstate, Millipore), Mcl-1 (clone S-19), Bcl-X_L (clone S-18) and Bcl-2 (clone 100) from Santa Cruz Biotechnology Inc. (Santa Cruz), and Bim (extra-large isoform) from Calbiochem (Darmstadt). HRP-labeled anti-mouse IgG (Sigma) and anti-rabbit IgG (Sigma and Cell Signaling Technologies) were used as secondary antibodies. Equal protein loading was confirmed by probing membranes with anti-β-actin or anti-α-tubulin antibodies (Sigma).

PIP_3 ELISA assay

PI3K activity was determined by measuring the amount of PIP_3 extracted from CLL cells, according to manufacturer's instructions (Echelon Biosciences). Briefly, PIP_3 were extracted from cell samples and incubated with a PIP_3 detector protein for 1 hour at room temperature. After this time, the mixture was transferred to the PIP_3-coated microplate for competitive binding and allowed to incubate 1 hour at room temperature.

Plates were washed and then incubated with a peroxidase-linked secondary detector for 45 minutes. 3,3',5,5'-tetramethylbenzidine colorimetric substrate was added for 5-10 minutes at which time reactions were stopped upon H_2SO_4 addition. The PIP_3 detector protein binding was analyzed at 450 nM. The colorimetric signal is inversely proportional to the amount of PIP_3 extracted from cells and cellular PIP_3 products were estimated using a standard curve. Results are displayed relative to untreated controls.

mRNA quantification by real-time PCR

Total RNA was extracted using TRIZOL method (Life technologies) according to manufacturer's instructions. One microgram of RNA was retrotranscribed to cDNA using random primers and the M-MLV reverse transcriptase (Life technologies). *BIM, MCL-1, CCL3* and *CCL4* mRNA expression levels were analyzed in duplicate with predesigned Assay-On-Demand probes (Life Technologies) on a Step-One Plus™ Real Time PCR System (Applied Biosystems) by quantitative real-time PCR (qRT-PCR). The relative expression of each gene was quantified by the comparative cycle threshold (C_t) method ($\Delta\Delta C_t$) using β-actin as endogenous control. mRNA expression levels are given in arbitrary units, taking as a reference the control sample (untreated cells).

RNA interference assay

CLL cells (2 x 10^6 cells/mL) were cultured without antibiotics and washed with FBS-free RPMI medium. 5 x 10^6 cells were then electroporated with a Nucleofector II device (Lonza) in 100 µL of Solution V (Lonza) containing either 5 µM of *BIM* Silencer Select Predesigned siRNA or 5 µM of a nonsilencing negative control (Life technologies). U-015 Nucleofector program was used. After transfection, cells were transferred to culture plates for 6 hours before experiments were set up.

BCR stimulation by anti-immunoglobulin M antibodies cross-linking

After 1.5 hours of starving in FBS-free RPMI 1640 culture medium, CLL cells (10^7 cells/mL) were reacted at 4ºC for 30 minutes with 25 µg/mL anti-IgM-biotin (Jackson Immunoresearch Laboratories) followed by the addition of 10 µg/mL streptavidin (Jackson Immunoresearch Laboratories) for 20 minutes. Cells were then transferred to 37ºC and collected after 30 minutes for further analysis. When indicated, cells were treated with NVP-BKM120 simultaneously to the addition of anti-IgM.

Stromal cell coculture

The human bone marrow-derived stromal cell line HS-5 (ATCC) was cultured in DMEM medium (Life technologies) supplemented with 10 % heat-inactivated FBS, 2 mM glutamine, 50 µg/mL penicillin-streptomycin (Life technologies). HS-5 cells were seeded in 24-well plates at 2 x 10^5 cells/mL the day before the experiment. The next day, medium was removed and CLL cells (2 x 10^6 cells, ratio 1:10) were added and cultured for the indicated times in the presence or absence of NVP-BKM120. CLL cells were collected by carefully rinsing the well without disturbing the HS-5 monolayer and then analyzed for cell viability.

Migration assays

CXCL12-induced migration was evaluated in 24-well chemotaxis plates containing transwell polycarbonate inserts of 6.5 mm diameter and 5 µM pore size (Corning). A total of 100 µL cell suspensions at 5 x 10^6 cells/mL pretreated or not with NVP-BKM120 for 1 hour were added to the top chamber of the transwells and 600 µL of RPMI 1640 with or without human recombinant CXCL12 (200 ng/mL; Peprotech) to the lower chamber. After 4 hours, 100 µL were collected in triplicate from each lower chamber and counted in a FACScan flow cytometer for 1 minute at constant flow rate.

Actin polymerization assays

CLL cells were serum-starved for 1.5 hours in FBS-free RPMI at 10^7 cells/mL. Then, cells were diluted to 2 x 10^6 cells/mL in RPMI with 0.5% BSA and treated with NVP-BKM120 for 1 additional hour. Samples were stimulated with 200 ng/mL of CXCL12 and at the indicated time points, 400 µL of the cell suspension were collected and added to 100 µL of the staining solution (2.5 ng/mL phalloidin-tetramethyl rhodamine isothiocyanate, 2.5 mg/mL of L-α-lysophosphatidylcoline (Sigma) and 5% paraformaldehyde (Aname) for 20 minutes at 37ºC. Samples were analyzed by acoustic cytometry (Attune; Life Technologies) and results were plotted relative to the mean fluorescence of the sample before the addition of CXCL12.

SUPPLEMENTARY TABLE

Table S1.- Characteristics of CLL patients

Patient nº.	Age at diagnosis	Gender	Source	Binet stage	Previous treatment	%CD19/ CD5†	IgVH status‡	%ZAP-70†	%CD38†	Cytogenetic alterations (FISH)§	Mutations‡	% response (2μM) 48h
CLL 1	53	M	PB	C	R-FCM, R-CHOP	97	UM	42	57	91% 13q del	normal	7.5
CLL 2	72	M	PB	A	No	93	M	0.3	0	18% 13q del	normal	55
CLL 3	51	M	PB	B	No	96	UM	17	63	94% 11q del	normal	55.2
CLL 4	73	M	PB	A	No	96	UM	67	1	44% 13q del	normal	45.4
CLL 5	73	M	PB	A	No	97	UM	9	8	normal	normal	28.6
CLL 6	46	M	PB	B	No	97	UM	5	1	38% 13q del; 15% 11q del; 25% 17p del	normal	23
CLL 7	63	F	PB	B/C	Chlorambucil	95	M	50	6	94% 13q del	normal	12.7
CLL 8	58	M	PB	A	No	95	M	8	3	normal	normal	23.2
CLL 9	64	M	PB	A	No	97	M	0.6	0.5	normal	normal	35.4
CLL 10	51	M	PB	B/C	FCM, R-FCM	93	M	13	53	normal	normal	26.3
CLL 11	57	M	PB	C	FCM	99	UM	77	50	97% 11q del	normal	16.5
CLL 12	72	F	PB	B/C	Yes	91	UM	55	100	85% 13q del	NOTCH1 mut	35
CLL 13	77	F	PB	B	FC	82	UM	73	46	90% 13q del; 80% 11q del	NOTCH1 mut	30
CLL 14	70	M	PB	A	R-FCM	93	M	0.8	7	normal	normal	59.9
CLL 15	60	M	PB	B	No	79	UM	35	40	75% 11q del	normal	34.8
CLL 16	76	M	PB	A	No	91	UM	27	14	normal	normal	34.3
CLL 17	70	F	PB	C	Fludarabine, chlorambucil	98	UM	34	3	normal	normal	38
CLL 18	72	M	PB	B	RFC	95	UM	7	10	93% 11q del; 78% 13q del	normal	36.9
CLL 19	82	M	PB	A	No	95	M	3	53	normal	normal	11
CLL 20	61	M	PB	A	FCM, CHOP-like, R-FCM	92	M	2	18	31% 13q del	SF3B1 mut	15.9
CLL 21	76	M	PB	B	Chlorambucil	93	M	5	70	normal	SF3B1 mut; NOTCH1 mut	23
CLL 22	60	M	PB	A	Chlorambucile, R-FCM	95	M	0	1	74% 13q del	normal	7.3
CLL 23	76	F	PB	A	Chlorambucil	86	M	26	44	90% 13q del	NOTCH1 mut	33.5
CLL 24	50	F	PB	A	No	95	M	25	0.4	70% 13q del	normal	28.1
CLL 25	55	M	PB	B	FCM, R-FCM	97	UM	40	44	89% 11q del	normal	31
CLL 26	54	M	PB	A	No	96	M	12	2	75% 13q del; 21% 17p del	MYD88 mut	39.7
CLL 27	75	M	PB	A	No	95	UM	18	20	12% 13q del	normal	8.5
CLL 28	57	F	PB	B	No	92	M	1	2	76% 13q del	normal	50.6
CLL 29	49	M	PB	A	No	89	M	2	10	28% 13q del	normal	26.3
CLL 30	69	M	PB	A	No	93	M	1	16	45% trisomy 12	normal	21.5
CLL 31	66	M	PB*	B	Chlromabucil, R-FCM	98	M	2	0.7	90% 13q del; 73% trisomy 12	normal	61.5
CLL 32	78	F	PB	A	No	94	M	1	0.2	18% 13q del	normal	33
CLL 33	83	M	PB	A	Chlorambucil	81	UM	0.8	0	19% 17p del	normal	17.6
CLL 34	67	M	PB	C	No	96	UM	7	0.6	90% 11q del	normal	20.6
CLL 35	74	M	PB*	B	CHOP-like	96	UM	24	50	68% trisomy 12	NOTCH1 mut	71.4
CLL 36	53	M	PB*	B	No	96	UM	7	95	normal	normal	26.2
CLL 37	46	M	PB	A	No	80	UM	70	30	34% 13q del	NOTCH1 mut	37.9

Abreviations: nd: not determined; M: male; F: female; PB: peripheral blood; BM: bone marrow; LN: lymph node; UM: unmutated; M:mutated; del: deletion; mut: mutation

† quantified by flow cytometry

‡ assessed by direct sequencing

* Bone marrow or lymph node samples were also assessed