A purine scaffold Hsp90 inhibitor destabilizes Bcl6 and has specific anti-tumor activity in Bcl6 dependent B-cell lymphomas

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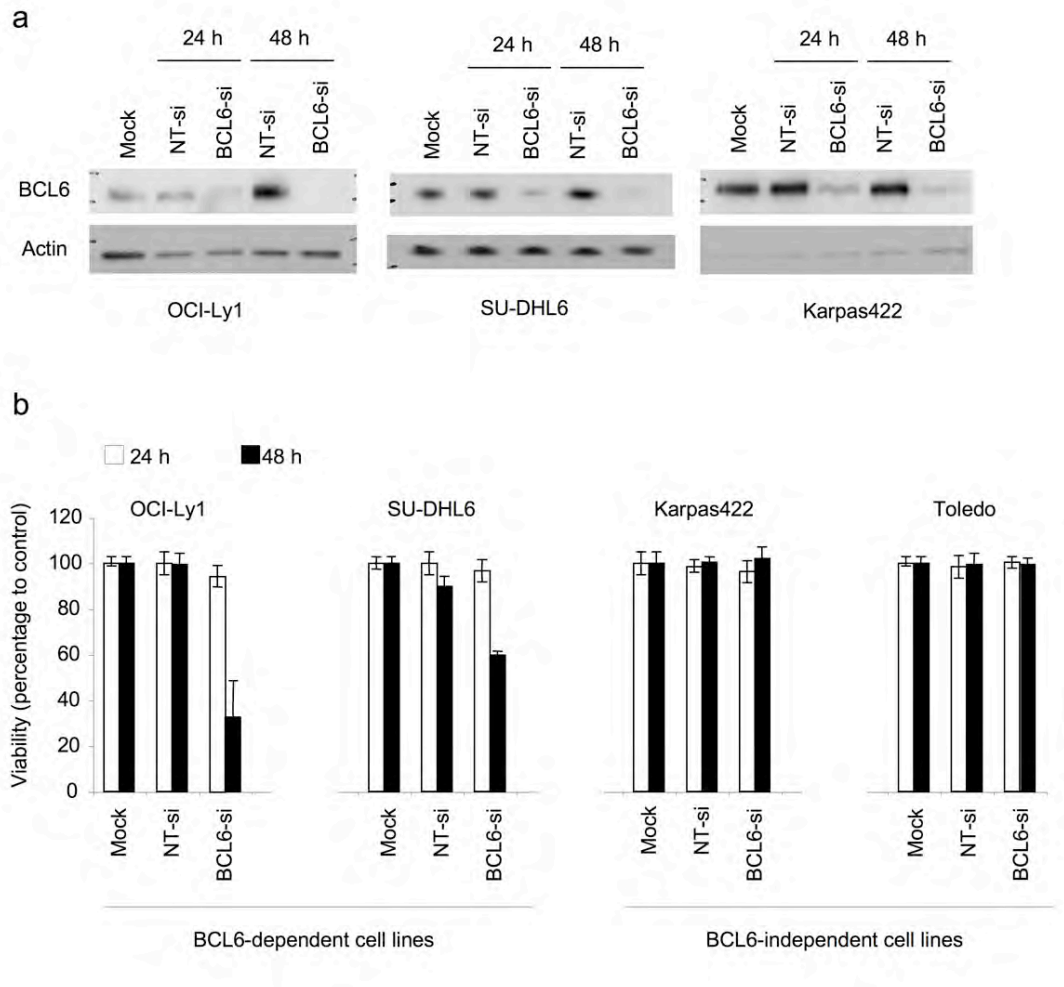
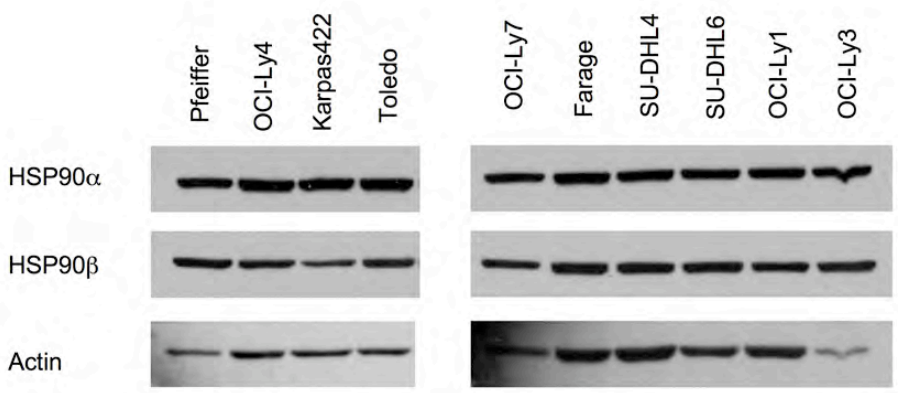
Supplementary Figures and Tables

Supplementary Figure 1



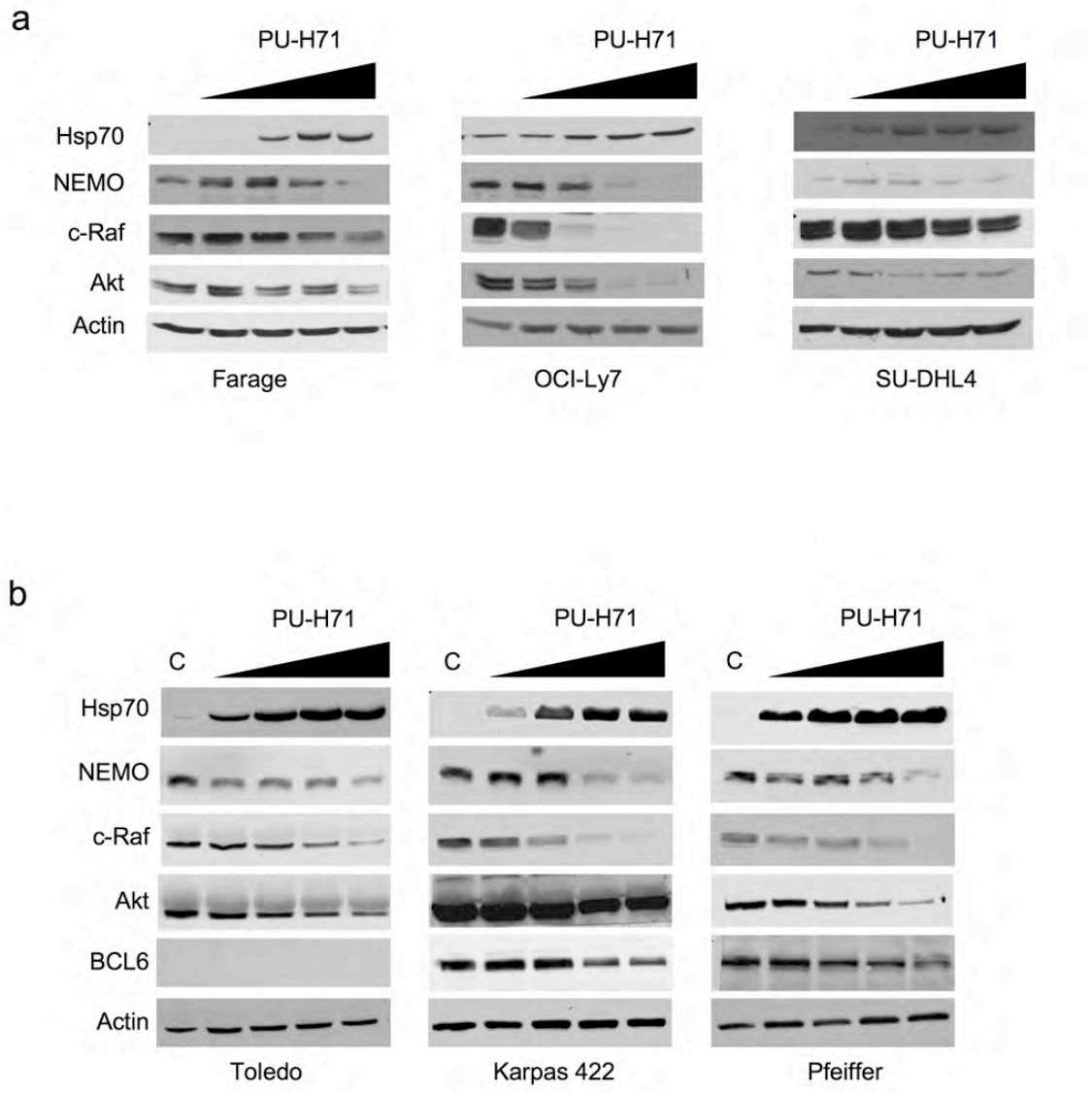
Supplementary Figure 1 Bcl6 siRNA kills Bcl6-dependent but not Bcl6-independent DLBLC cells. OCI-Ly1, SU-DHL6, Karpas422 and Toledo cell lines were transfected with non-targeted siRNA (NT-si), BCL6 siRNA (BCL6-si) or nothing (Mock). (**a**) Bcl6 protein abundance was determined by immunoblotting 24 and 48 h after the transfection in all the Bcl6-postive cell lines (i.e.: not in Toledo cells since they do not express Bcl6 – see **Supplementary Figure 3B**). (**b**) Cell viability was determined by acridine orange/ethidium bromide dye exclusion at the same time-points. Results represent the average of 3 or 4 replicates and are expressed as percentage of viable cells to control (mock at 24 and 48 h respectively). OCI-Ly1 and SU-DHL6 are Bcl6-dependent cell lines while Karpas422 and Toledo are Bcl6-independent cell lines.

Supplementary Figure 2



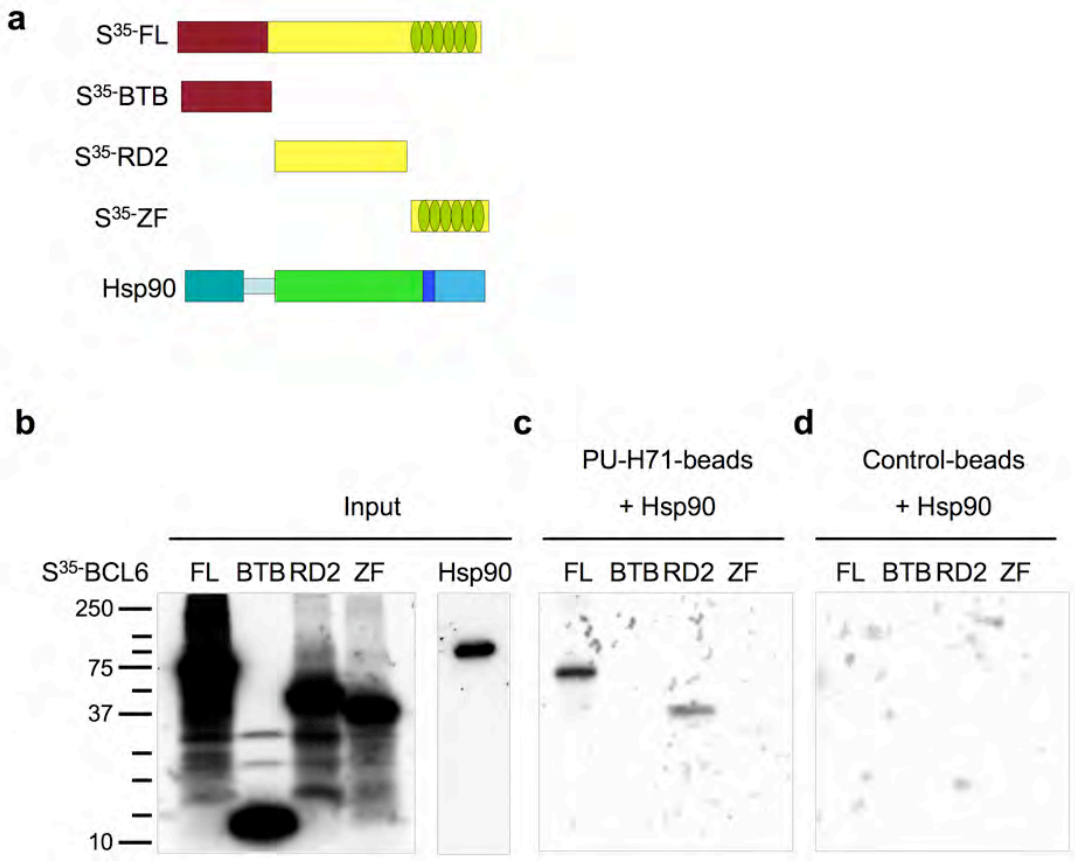
Supplementary Figure 2 Expression of the Hsp90-α and Hsp90-β isoforms in DLBCL cell lines. Western blotting was performed to detect Hsp90-α and Hsp90-β in a panel of DLBCL cell lines. Densitometry results for this experiment are included in **Supplementary Table 1**. Actin western blots were included as loading control.

Supplementary Figure 3



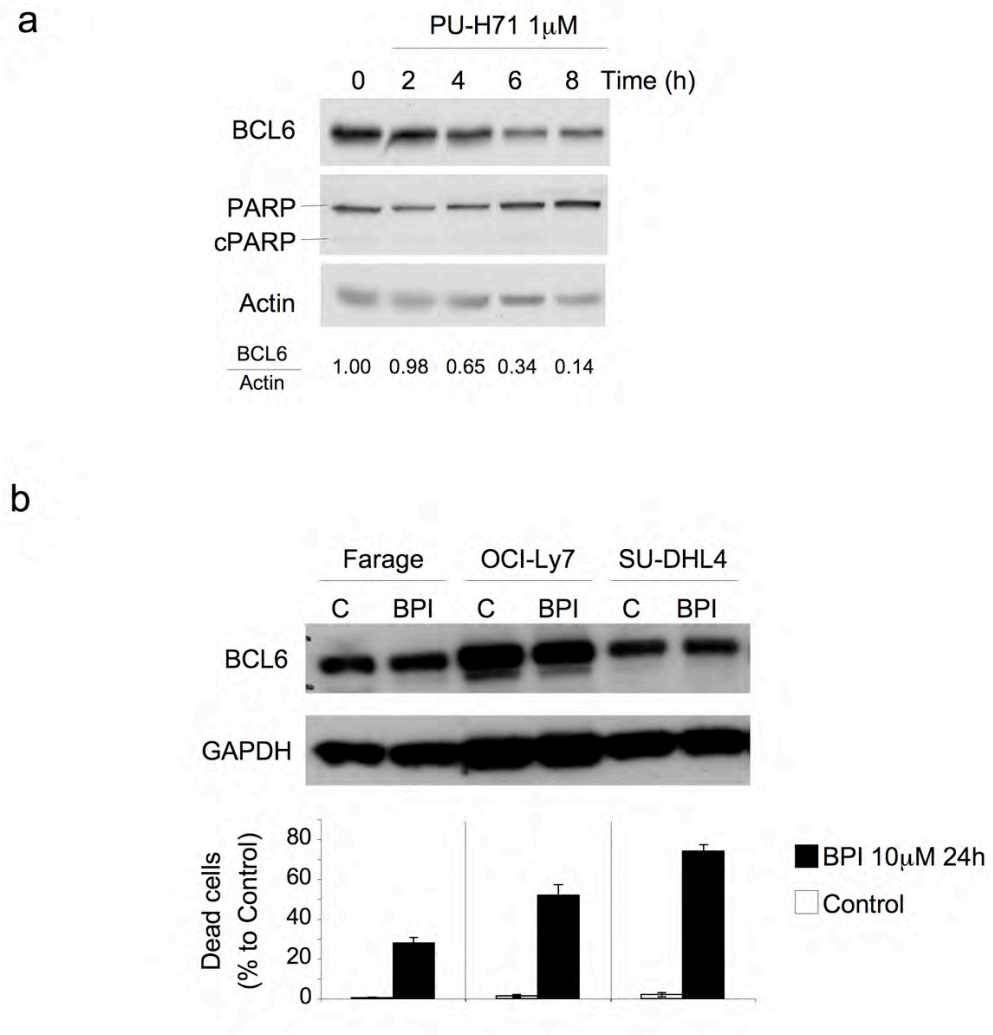
Supplementary Figure 3 The impact of PU-H71 on Hsp90 client proteins in Bcl6 dependent and independent DLBCL cell lines. (**a**) Western blotting was performed for the Hsp90 client proteins NEMO, c-Raf and Akt and for Hsp70 in Farage, OCI-Ly7 and SU-DHL4 cell lines exposed for 24 h to increasing concentrations of PU-H71 (0.1, 0.25, 0.5 and 1 μM). Actin western blots were performed for loading control. (**b**) Western blotting was performed for Bcl6, the known Hsp90 client proteins NEMO, c-Raf and Akt and for Hsp70 in the Bcl6-independent Toledo, Karpas422 and Pfeiffer DLBCL cells exposed for 24 h to increasing concentrations of PU-H71 (0.1, 0.25, 0.5 and 1 μM). Actin western blots were performed as loading control.

Supplementary Figure 4



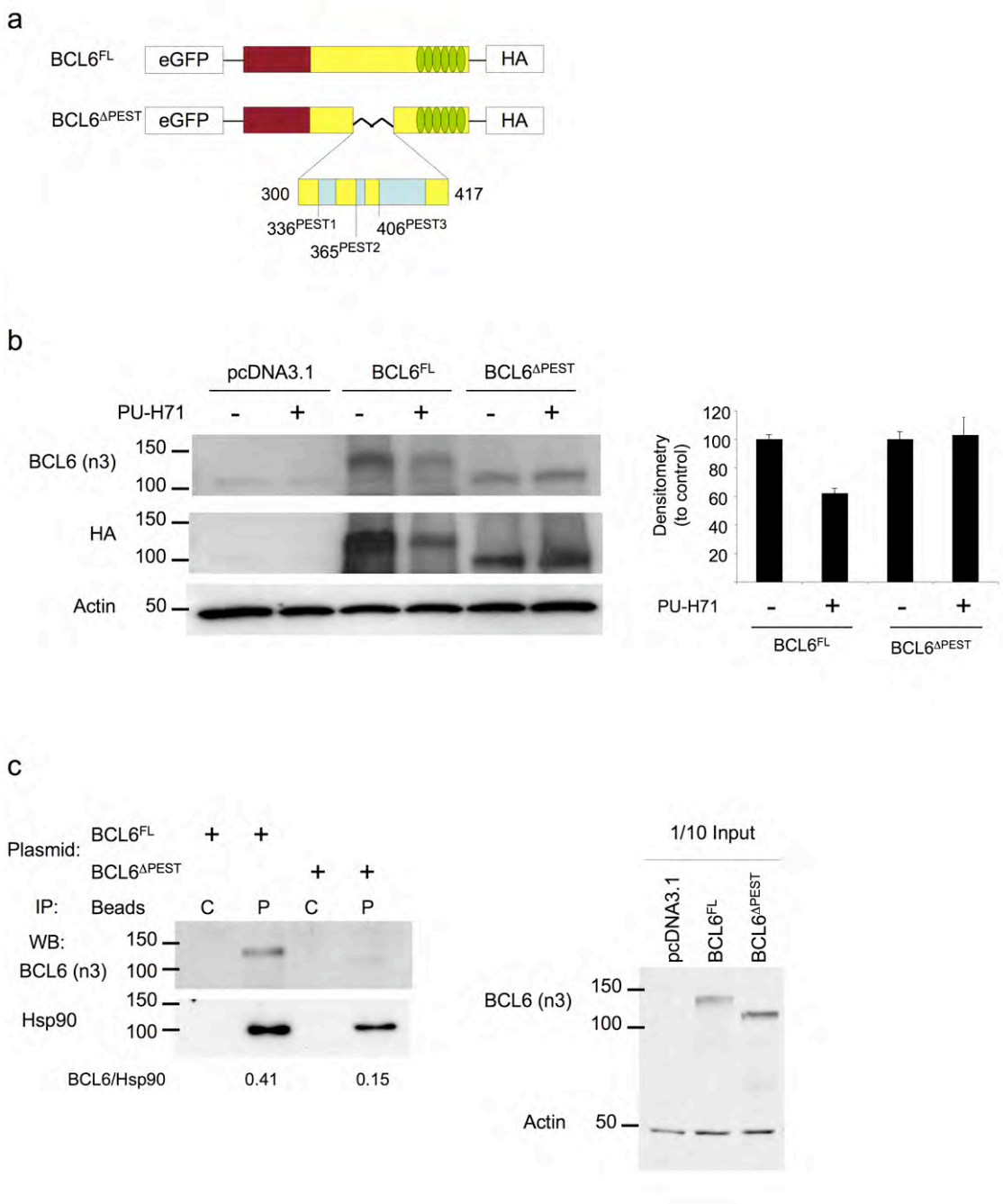
Supplementary Figure 4 Hsp90 binds to the Bcl6 middle repression domain. (**a,b**) We first generated *in vitro* translated S^{35}-labelled full-length (S^{35}-FL) BCL6 and fragments corresponding to the 3 domains of BCL6: S^{35}-BTB, S^{35}-RD2 (middle domain), S^{35}-ZF, for the BTB/POZ-domain, middle region and zinc-finger domains respectively. In vitro translated Hsp90 was also generated and confirmed by western blotting with Hsp90 antibody. The Hsp90 sample was split into four equal fractions and mixed with the various Bcl6 proteins and then subjected to protein affinity chromatography using PU-H71-bound or control agarose beads containing only the chemical linker. The ability of PU-H71 or control beads to capture the various fragments was then resolved by SDS-PAGE and fluorography. (**c,d**) Only full length Bcl6 and the Bcl6 RD2 interacted with Hsp90. The input represents the direct loading of the S^{35}-labeled proteins. The figure shows one of four replicate experiments.

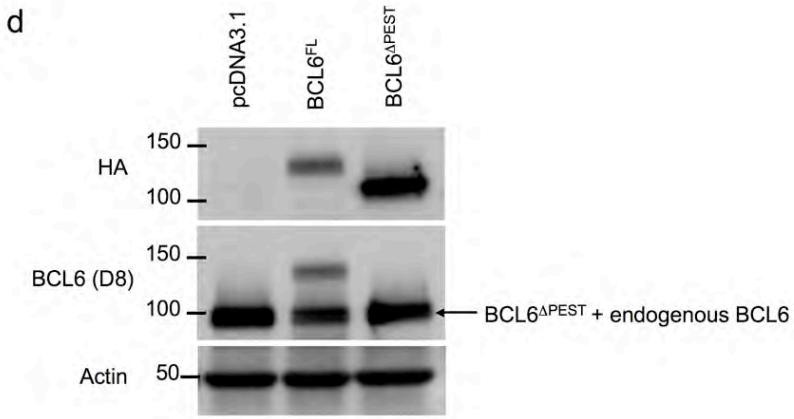
Supplementary Figure 5

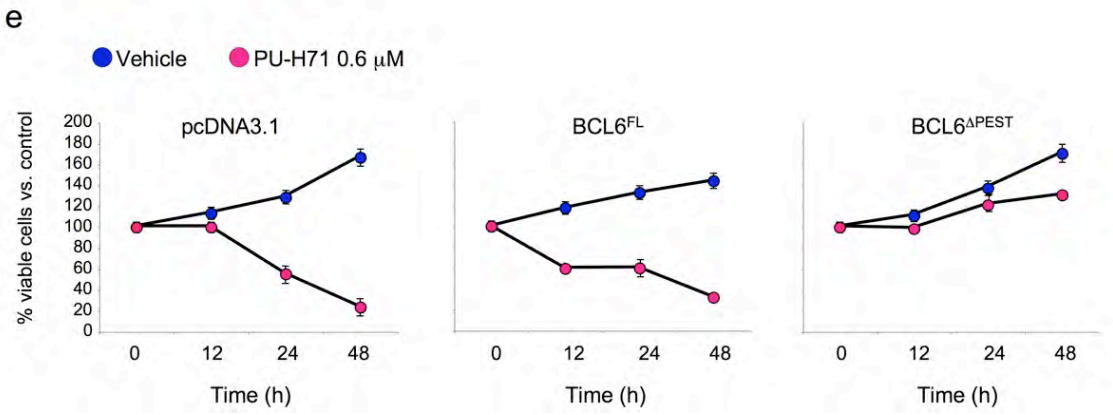


Supplementary Figure 5 Bcl6 degradation precedes apoptosis in DLBCL cells and is not a non-specific event associated with cell death. (**a**) Cell extracts were submitted to western blotting with antibodies specific for Bcl6, PARP and actin at various times after exposure to PU-H71 in OCI-Ly7 DLBCL cells. Actin was used as loading control. Densitometry values for the ratio BCL6/actin are shown at the bottom. These data show that Bcl6 degradation occurs rapidly in DLBCL cells and precedes the occurrence of apoptosis as indicated by the lack of PARP cleavage at these early time points. (**b**) Farage, OCI-Ly7 and SU-DHL4 were exposed to 10 μM Bcl6 retro-inverso peptidomimetic inhibitor (BPI) or control peptide (C) for 24 h. Western blots were performed to detect Bcl6 or GAPDH (as a loading control). The percentage of dead cells under the same conditions was measured by the acridine orange/ethidium bromide dye-exclusion method in experimental triplicates. The data show that induction of cell death in Bcl6 dependent cell lines is not accompanied by Bcl6 degradation.

Supplementary Figure 6



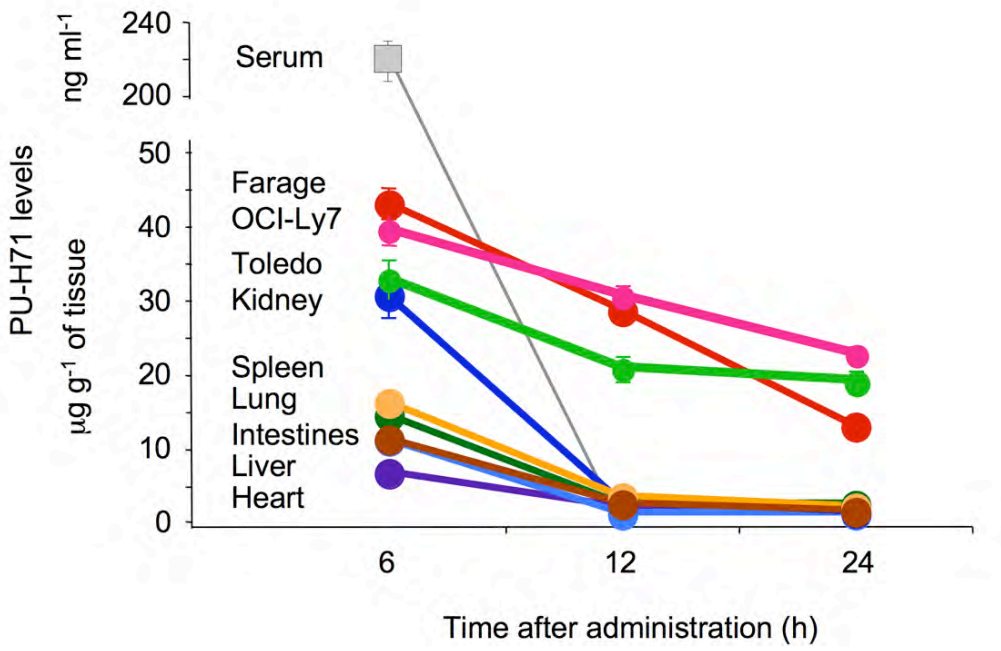




Supplementary Figure 6 Bcl6 degradation is required for PU-H71 induced cell death. (**a**) A cartoon representation of the plasmid constructs coding the full-length ($BCL6^{FL}$) and PEST-truncated ($BCL6^{\Delta PEST}$) Bcl6 proteins. (**b**) NIH-3T3 cells were transfected with empty vector (pCDNA 3.1), $BCL6^{FL}$ or $BCL6^{\Delta PEST}$ and then exposed to PU-H71 2.5 μM for 24 h. Immunoblotting was then performed using Bcl6 (N3), HA antibodies. Actin was used as loading control. Densitometry analysis is shown on the right and shows that PU-H71 induced degradation of $BCL6^{FL}$ with far greater efficiency than the $BCL6^{\Delta PEST}$. (**c**) The lysates of NIH-3T3 cells transfected with $BCL6^{FL}$ or $BCL6^{\Delta PEST}$ were subjected to protein affinity chromatography using PU-H71-coated agarose beads (P) or control agarose beads (C), followed by western blotting for Bcl6 (N3) and Hsp90. Hsp90 was observed to form a complex more readily with $BCL6^{FL}$ than $BCL6^{\Delta PEST}$ as underlined by the densitometry values (Bcl6/Hsp90) shown at the bottom. To the right are shown western blots of 1/10 of the input using Bcl6 (N3) antibodies and actin. (**d**) Farage (a Bcl6 dependent DLBCL) cells were transfected with empty vector (pCDNA 3.1), $BCL6^{FL}$ or $BCL6^{\Delta PEST}$ followed by western blot with HA antibody (to detect the HA-containing plasmids $BCL6^{FL}$ and $BCL6^{\Delta PEST}$) and a monoclonal Bcl6 antibody (D8, to detect the endogenous and

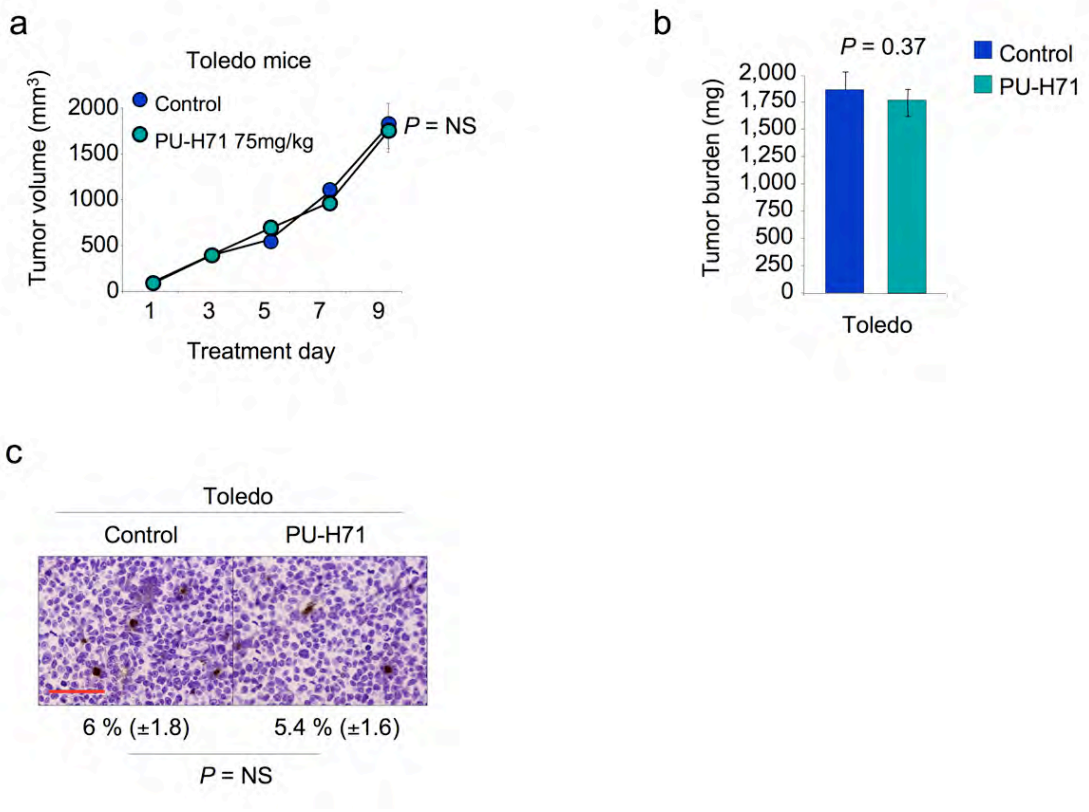
plasmid-coded Bcl6 proteins). Actin immunoblot was performed as loading control. Since the GFP-BCL6$^{\Delta PEST}$ is of the same molecular weight as endogenous Bcl6 the two bands cannot be distinguished in the Bcl6 (D8) western blot as indicating in the figure. (**e**) Farage cells transfected as in panel (d) were exposed to PU-H71 0.625 μM (approximate the GI_{50} for this cell line, red circles) or vehicle (control, blue circles). The number of viable cells was determined by CellTiter-Glo and acridine orange/ethidium bromide at baseline and then at 12, 24, and 48 h as indicated. The percent of viable cells as compared to cells at t = 0 is shown on the Y-axis. Experiments were carried out in triplicates with biological duplicates. PU-H71 caused a reduction in viability of empty vector and full length BCL6 transfected Farage cells, but had minimal effects on BCL6$^{\Delta PEST}$ transfected cells. Of note, these data show that the BCL6$^{\Delta PEST}$ transfected cells continued to expand even in the presence of PU-H71.

Supplementary Figure 7



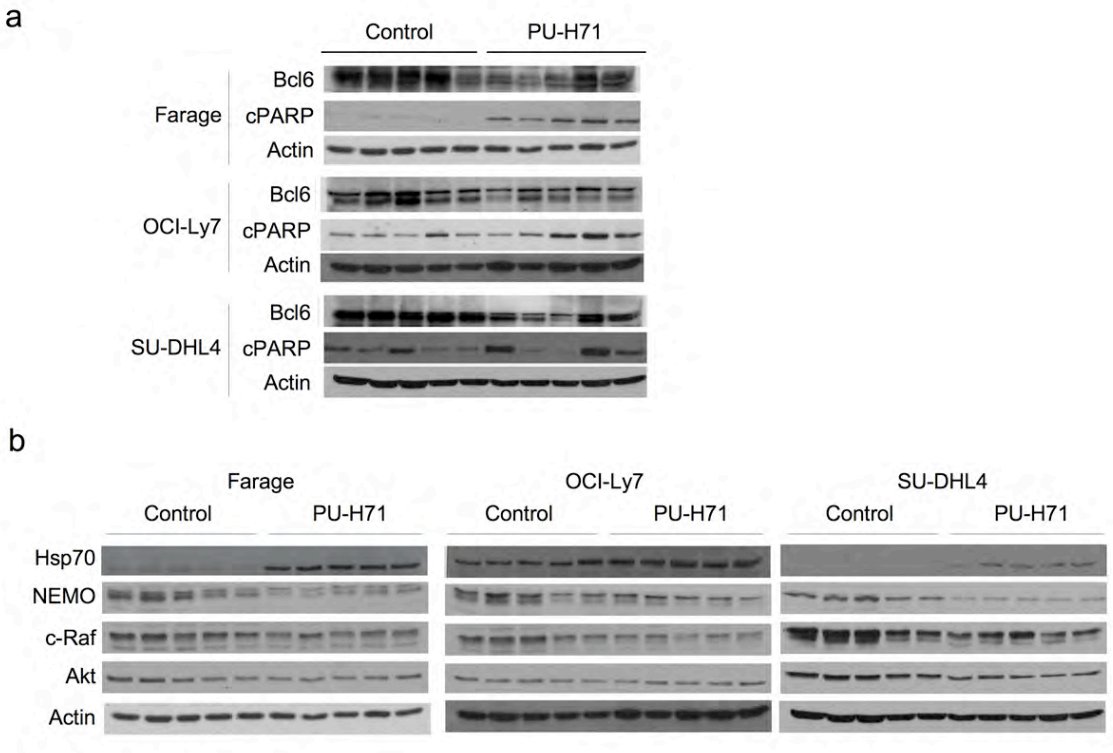
Supplementary Figure 7 PU-H71 is preferentially retained in DLBCL xenografts. PU-H71 levels were measured in serum (in ng ml^{-1}), Farage, OCI-Ly7 and Toledo DLBCL xenografts and normal tissues (in Farage only) (in µg per gram of tissue) by HPLC-MS/MS (Y-axis) at the indicated time points (X-axis) after a single dose of 75 mg per kg body weight. Measurements were carried out in duplicates.

Supplementary Figure 8



Supplementary Figure 8 PU-H71 does not suppress the growth of BCL6 independent DLBCL xenografts. (**a**) Tumor growth plots in Toledo xenografted mice (*n* = 10) treated with control (blue circles) or PU-H71 at 75 mg per kg body weight per day (green circles) for 10 consecutive days. The Y-axis indicates tumor volume (in mm^3) and X-axis days of treatment. The *P* value representing the comparison of tumor volumes at day nine by T-test was not significant. (**b**) Tumor burden (in mg) at day 10 in control (blue bars) and PU-H71 at 75 mg per kg body weight per day (green bars) treated Toledo mice. (**c**) TUNEL assay for the tumors from panel (a). Bar represents 100 μm.

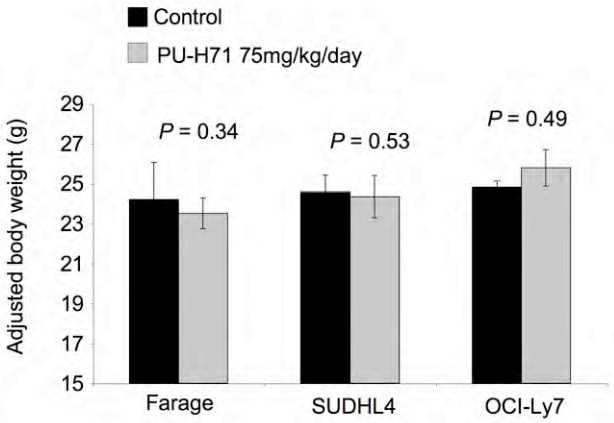
Supplementary Figure 9



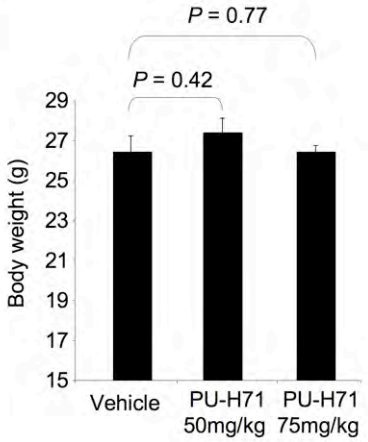
Supplementary Figure 9 The effect of PUH71 on PARP cleavage and client protein expression in DLBCL xenografts. (**a**) The abundance of Bcl6, cleaved-PARP (cPARP) and actin (as control) was determined by immunoblotting in lysates from Farage, OCI-Ly7 and SU-DHL4 xenografts. (**b**) The same lysates were also subjected to western blot for Hsp70, NEMO, c-Raf, Akt and Actin. The densitometry of these western blots is shown in Figures 5 and 6 in the main text.

Supplementary Figure 10

a



b

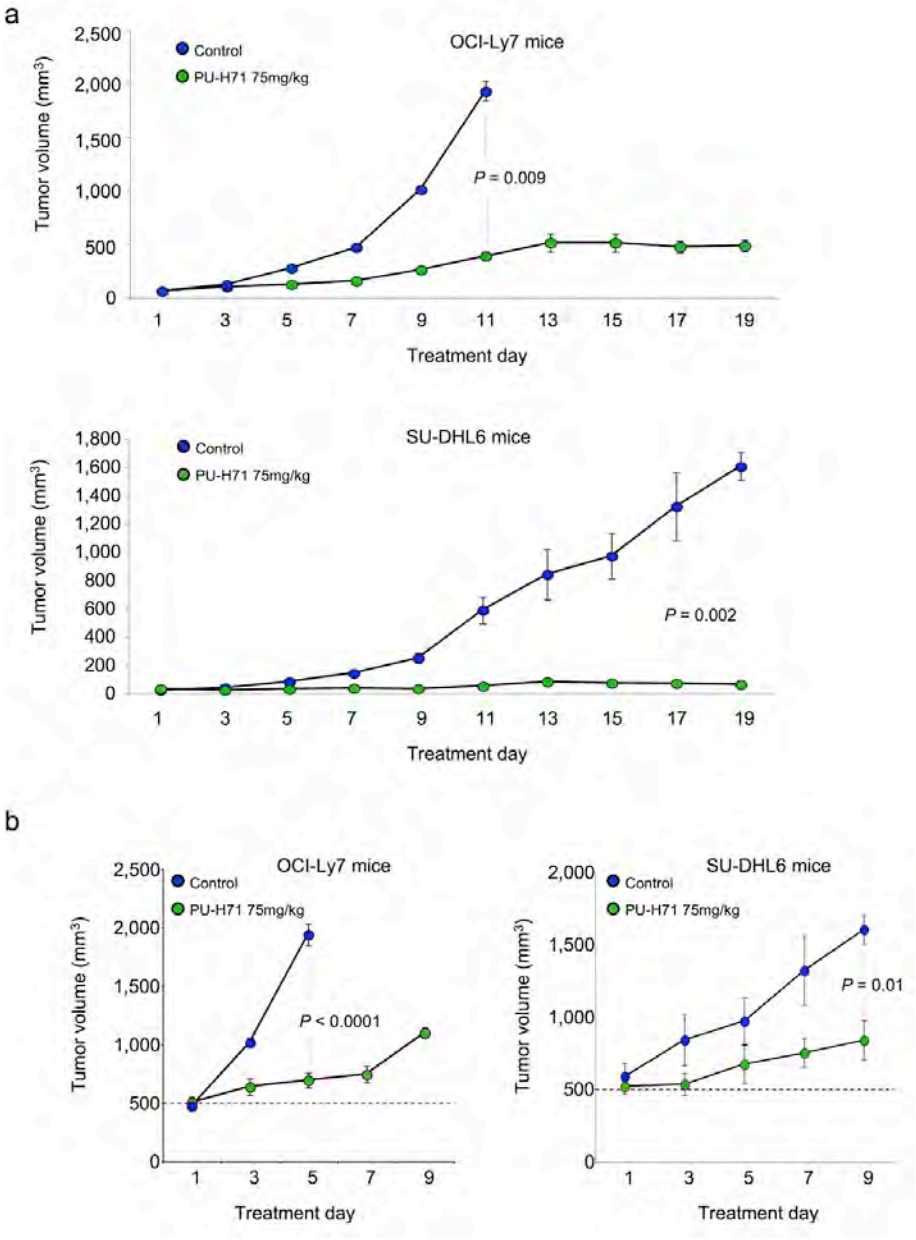


c

	Control vehicle 0.2 ml/day (*n* = 5)	PU-H71 50 mg/kg/day (*n* = 5)	PU-H71 75 mg/kg/day (*n* = 5)	Reference values	Units
WBC	5.16 ± 1.20	5.98 ± 1.51	5.82 ± 1.51	6.4 ± 2.5	K/μL
RBC	8.99 ± 0.80	8.92 ± 0.62	8.91 ± 0.62	8.53 ± 0.5	M/μL
Hemoglobin	13.60 ± 1.70	12.63 ± 1.47	13.02 ± 1.70	14.5 ± 1.1	g/dL
Platelets	278.00 ± 90.20	279.75 ± 89.44	256.30 ± 102.30		K/μL
ALP	92.40 ± 14.05	112.40 ± 16.10	116.00 ± 4.29	23-181	IU/L
ALT (SGPT)	99.80 ± 83.23	43.40 ± 44.55	72.00 ± 28.07	16-58	IU/L
AST(SGOT)	97.60 ± 37.70	64.60 ± 26.83	94.80 ± 30.66	36-102	IU/L
GGT	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0-2	IU/L
Albumin	2.60 ± 0.11	2.56 ± 0.13	2.54 ± 0.18	2.5-3.9	g/dL
Total Protein	4.72 ± 0.20	4.88 ± 0.17	4.64 ± 0.15	4.1-6.4	g/dL
Globulin	2.12 ± 0.11	2.32 ± 0.10	2.10 ± 0.06	1.3-2.8	g/dL
Total Bilirubin	0.20 ± 0.00	0.20 ± 0.00	0.24 ± 0.08	0-0.3	mg/dL
Blood Urea Nitrogen	21.60 ± 0.78	20.00 ± 1.64	19.20 ± 2.35	14-32	mg/dL
Creatinine	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.1-0.6	mg/dL
Cholesterol	112.20 ± 4.94	114.60 ± 5.63	102.20 ± 3.99	74-190	mg/dL
Glucose	177.20 ± 28.02	185.00 ± 19.51	197.60 ± 19.72	76-222	mg/dL
Calcium	8.56 ± 0.27	8.44 ± 0.33	8.26 ± 0.34	7.6-10.7	mg/dL
Phosphorus	9.10 ± 1.62	8.84 ± 0.66	7.26 ± 0.93	4.6-9.3	mg/dL
TCO2	19.40 ± 2.20	18.00 ± 2.48	18.20 ± 3.74	11-18	mEq/L
Chloride	110.00 ± 1.07	109.80 ± 2.27	110.40 ± 2.37	103-115	mEq/L
Potassium	4.94 ± 1.36	4.10 ± 0.09	4.10 ± 0.19	3.4-5.5	mEq/L
Sodium	153.20 ± 1.14	151.40 ± 1.18	148.20 ± 0.73	146-155	mEq/L
Osmolality	311.68 ± 3.47	306.66 ± 3.21	301.10 ± 1.02	300-330	mOsm/kg
Total T4	4.90 ± 0.46	4.52 ± 1.24	4.42 ± 0.62		μg/dL

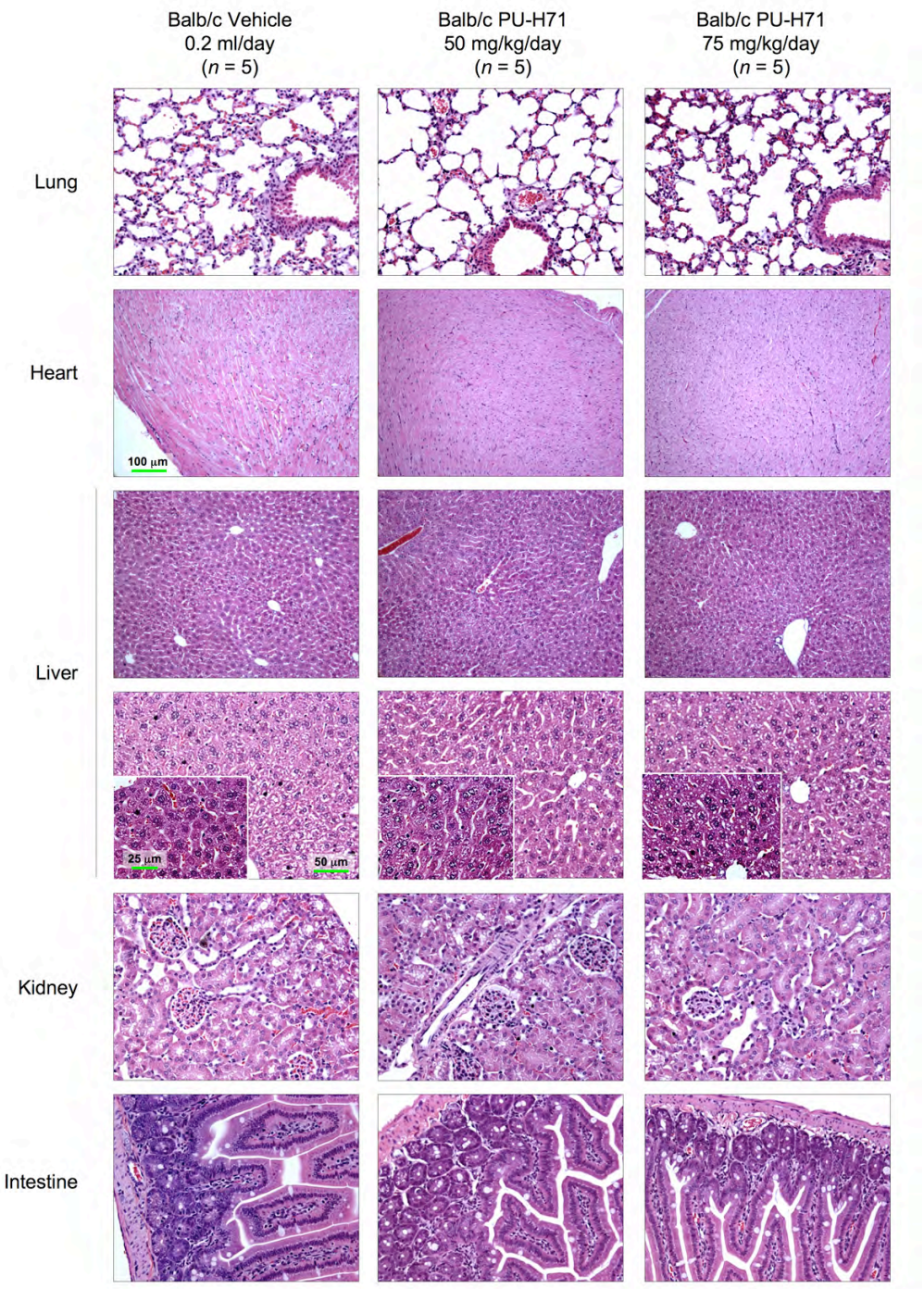
Supplementary Figure 10 Anti-lymphoma doses of PUH71 are non-toxic in mice (**a**) Adjusted (total-tumor) body weight in grams of the Farage, SU-DHL4 and OCI-Ly7 xenografted mice treated with control (5 mice per cell line) or PU-H71 75 mg per kg body weight per day (5 mice per cell line) for 10 days. *P* values were calculated using T-test. (**b**) Body weight (in grams) of 15 Balb/c mice treated with vehicle (*n* = 5), PU-H71 50 mg/kg/day (*n* = 5), and PU-H71 75 mg/kg/day (*n* = 5) for 10 consecutive days. *P* values were calculated using T-test. (**c**) Differential blood cell count, chemistry panel and T4 values from the mice treated as in panel (b).

Supplementary Figure 11



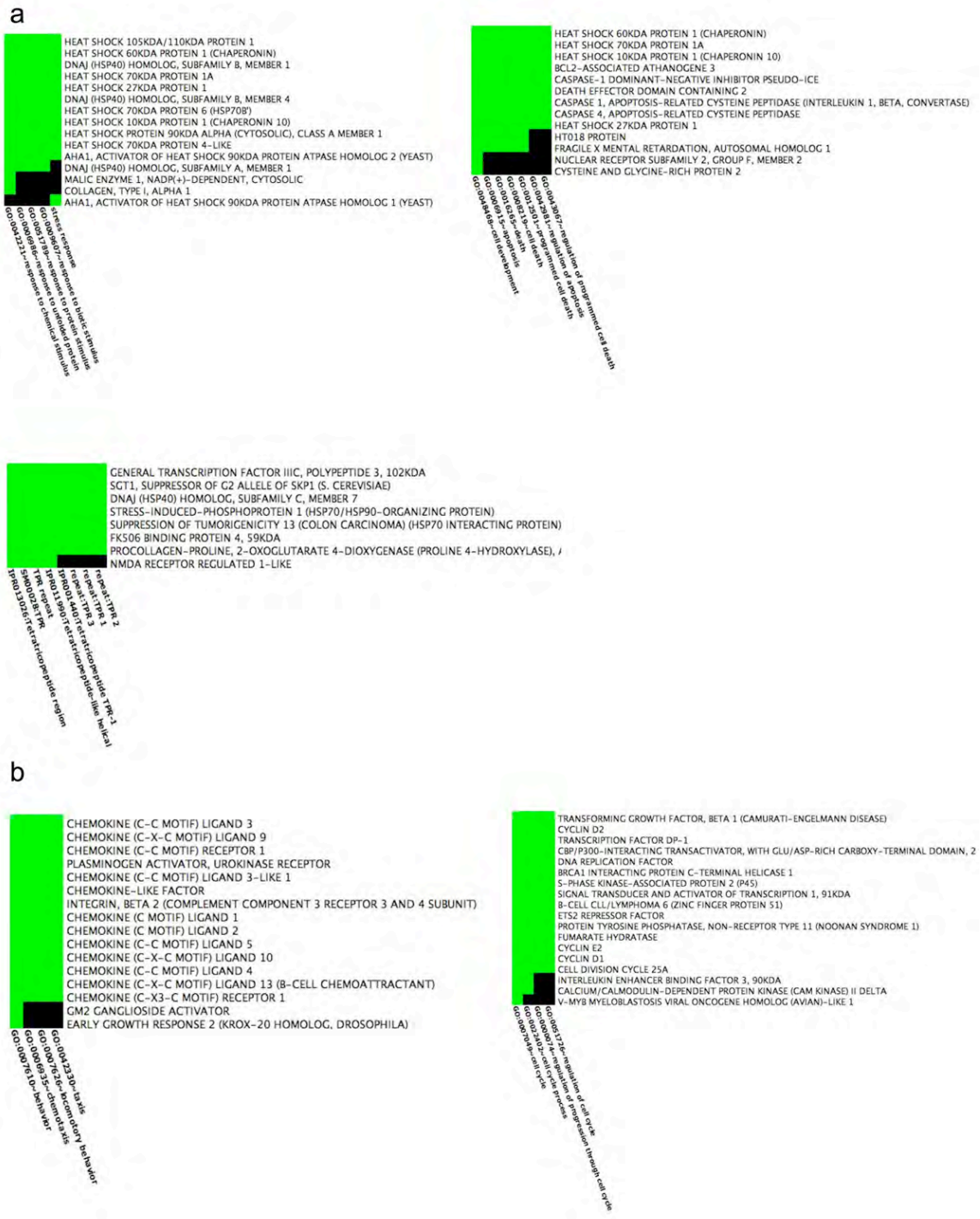
Supplementary figure 11 The anti-lymphoma impact of longer exposure to PU-H71 and the effect of PU-H71 on larger tumors. (**a**) Tumor growth plots in OCI-Ly7 (*n* = 10) and SU-DHL6 (*n* = 10) xenografted mice treated with control (blue circles) or PU-H71 at 75 mg per kg body weight per day (green circles) for 20 consecutive days. Treatment started with 75–100 mm^3 tumors in size. The Y-axis indicates tumor volume (in mm^3) and X-axis days of treatment. (**b**) Tumor growth plots in OCI-Ly7 (*n* = 10) and SU-DHL6 (*n* = 10) xenografted mice treated with control (blue circles) or PU-H71 at 75 mg per kg body weight per day (green circles), except treatment in this case was initiated when tumors reached 500 mm^3 in size (instead of 100 mm^3). *P* values were obtained by two-tailed T-test at the indicated time points.

Supplementary Figure 12



Supplementary Figure 12 Absence of changes in tissue architecture in mice exposed to PU-H71. Representative microscopic images (hematoxylin & eosin staining) of the tissues harvested from 15 Balb/c mice treated with vehicle (*n* = 5), PU-H71 50 mg per kg body weight per day (*n* = 5), and PU-H71 75 mg per kg body weight per day (*n* = 5) for 10 consecutive days.

Supplementary Figure 13



Supplementary Figure 13 Gene Ontogeny (GO) terms represented among PU-H71 responsive genes. Selected GO categories obtained using the DAVID gene ontogeny program for up-regulated (**a**) and down-regulated (**b**) genes from three pairs of Farage xenograft-bearing mice treated with 75 mg per kg body weight PU-H71 for 6 and 12 h v control (0 h).

Supplementary Table 1

	PUH71	17-DMAG	Bcl6	COO	*BCL2*	*TP53*	Hsp90-α	Hsp90-β
OCI-Ly4	100*	100*	IND	GCB	rearrangement	mut	2.8	2.3
Karpas422	100*	100*	IND	GCB	t (14,18)	wt	3	1.8
Toledo	75*	68*	IND	GCB	wt	*NA*	3.6	2.6
Pfeiffer	10.64	15.6	IND	*NA*	t (14,18)	mut	3.8	4.6
OCI-Ly10	3.95	1.77	DEP	ABC	wt	wt	3.2	3.8
SUDHL4	2.56	0.63	DEP	GCB	t (14,18)	mut	2.3	2.4
OCI-Ly3	1.25	4.12	DEP	ABC	amplification	wt	6.6	6.2
Farage	0.74	0.5	DEP	GCB	wt	mut	2.7	2.6
OCI-Ly1	0.6	0.68	DEP	GCB	t (14,18)	mut	2.9	2.5
SU-DHL6	0.39	0.33	DEP	GCB	t (14,18)	wt	3.4	3.6
OCI-Ly7	0.23	0.32	DEP	GCB	-18q 12-23	mut	2.8	2.3

Supplementary Table 1 GI_{50} values for PU-H71 (in μM) and 17-DMAG (in μM), along with molecular and cellular features for the panel of DLBCL cell lines used in our experiments**.**

IND: independent, DEP: dependent, wt: wild type, mut: mutant, GCB: germinal center B-cell, ABC: activated B-cell, NA: not available. Numbers for Hsp90 represent densitometry values from western blots. *Indicates that the value is estimated using the Chou-Talalay equation and not from actual experiments using that concentration (values equal or higher than 100 μM are shown as 100 μM).

Supplementary Table 2

a

Down-regulated genes

ABCG1	CXCL9	IQGAP1	SAMHD1
ADO	CYP51A1	ITGB2	SCAMP1
AGBL3	DDX42	ITPR1	SCD
AK2	DHX9	JAKMIP1	SEC61A1
ANKRD22	DIDO1	KCNIP2	SEMA4D
ANP32A	DIRAS2	KCTD12	SFRS10
AP1S2	DPY19L2P2	KIAA0372	SKP2
AP1S3	DRAM	KIAA0430	SLAIN2
APOL6	EGR2	KLF10	SLC38A5
ARHGDIA	EGR3	LITAF	SLC4A7
B3GALNT1	EHBP1L1	LMNA	SMC3
B4GALT1	EHD3	TAF9B	SMG1
BCL6	EHD4	LRRC58	SNHG4
BHLHB2	ELK3	MAFF	SNX5
BHLHB3	ELOVL6	MATR3	SOCS3
BPGM	ENO1	MAZ	SPATA5L1
BRIP1	ERAP2	MBNL1	SQLE
BTLA	ERF	MCAM	SRPR
C10orf76	ETV7	MCL1	ST8SIA4
C13orf15	EZR	MCM3AP	STAT1
C18orf19	FAM101B	MCM4	SUB1
C1orf121	FAM55C	MCOLN2	SUPT16H
C20orf27	FAM62B	MEX3D	TANK
C20orf67	FBXO6	MFNG	TBX21
C5orf24	FERMT3	MRTO4	TFDP1
CAMK2D	FGD6	MTAP	TFEC
CASP8	FH	MTMR1	TFRC
CCDC85B	FIP1L1	MYBL1	TGFB1
CCL3	FLJ20160	NSMAF	TLR7
CCL4	FSCN1	NUPL1	TMEM5
CCL5	FUS	OPRS1	TNFRSF1B
CCND1	FUT7	P4HB	TOR1AIP1
CCND2	FVT1	PICALM	TOX2
CCNE2	GART	PLAUR	TPM4
CCR1	GATC	PLEKHA2	TTF2
CD44	GBP1	PPIA	TXK
CDC25A	GBP2	PRDX2	TXNDC1
CDT1	GBP5	PRKXP1	UBD
CDV3	GLDC	PSME4	UGCG
CEBPB	GLUD2	PSPC1	UGT2B17
CHST11	GM2A	PTPN11	UNC93B1
CITED2	GNLY	PURB	USP1
CKLF	HHEX	RAB31	USP12
CLECL1	HLA-DRB1	RALGPS2	VPS35
CMAH	IDS	RASA4	WAC
CREB5	IFI44L	RBM14	WDR1
CTDSPL2	IFIT3	RERE	WDR76
CUGBP2	IKZF1	RGS16	WSB2
CX3CR1	IL32	RRAGD	XCL1
CXCL10	ILF3	RWDD2B	YWHAE
CXCL13	IMPACT	S100A4	ZBED2

b

Up-regulated genes

ABCB9	HSPA6
AHSA1	# HSPB1
AHSA2	HSPD1
ATP2C1	HSPE1
BAG3	HSPH1
C1orf96	LGMN
C6orf60	LOC284837
# CACYBP	MBNL2
CASP1	ME1
CASP4	# MME
CBFA2T3	MUM1
CCDC117	NARG1L
CCDC4	NR2F2
CHORDC1	NUDT9
CLIC2	P2RX5
COL1A1	P4HA2
COP1	PCBD1
CRYZ	PLOD1
CSRP2	PRKACB
CTSLL3	PRO1073
CXCR7	PSIP1
DEDD2	PTOV1
DNAJA1	RARA
DNAJB1	RNF144B
# DNAJB4	SEMA6A
DNAJC7	SEPW1
EGFR	SHOX2
EHF	SLC5A3
FANK1	SNORA71B
FCRLB	SPAG5
FKBP4	# ST13
FXR1	STAT4
GKAP1	STIP1
GSN	SUGT1
GTF3C3	SYTL4
HBE1	TSHZ1
HSP90AA1	TSPYL4
# HSPA1A	# UBR2
# HSPA1B	ZC3HAV1
HSPA4L	

Supplementary Table 2 Gene expression changes induced by PU-H71. Complete list of the 204 down-regulated (**a**) and 79 up-regulated (**b**) unique genes from the Farage mice treated with 75 mg/kg PU-H71. In red are the genes that contain at least one ARE domain in the 3' UTR region. Obtained from the ARE database and manually curated. (#) denotes up-regulated ARE-containing genes that are related to the heat shock-ubiquitin-proteasome pathway.

Supplementary Table 3

Rank	ID	Name	C-map score
1	972	GA	1
2	1005	17-AAG	0.973
3	947	17-AAG	0.968
4	1006	17-AAG	0.934
5	1063	17-AAG	0.901
6	953	monorden	0.896
7	1066	GA	0.886
8	1064	17-AAG	0.885
9	836	monorden	0.884
10	1051	17-DMAG	0.883

Supplementary Table 3 Connectivity map results for the first 10 ranked perturbagens obtained with the PU-H71 gene expression signature.

GA: geldanamycin.

Supplementary Table 4

Gene	Primer sequence (5'– 3')
BCL6	GACTCTGAAGAGCCACCTGC CTGGCTTTTGTGACGGAAAT
TP53	TCTTCTGTCCCTTCCCAGAA AATCAACCCACAGCTGCAC
ATR	AAGCGCCACTGAATGAAACT GTCGCTGCTCAATGTCAAGA
GAPDH	CGACCACTTTGTCAAGCTCA CCCTGTTGCTGTAGCCAAAT
HPRT	GCACACAGAGGGCTACAATGTG CGTCTTGCTCGAGATGTGATG
B2M	GCTTACATGTCTCGATCCCACTT GCCGTGTGAACCATGTGACT
CCND2	CCCTCTGCTGAGCGGTACTAA TCTTATCCTGCCAATTCAGTGTGA
HSAP90A	ACCAAAGAAGGCCTGGAACT CATGGAGATGTCACCAATCG
HSP90B	GAAACCGCCCTGCTATCTTC GGGGATCTCATCAGGAACTG
CDC37	ATGTGAAGGACGTGCAGATG AGAGGCCAGAGTCAATGCAG
AHSA1	TGAAGTGGAGGTTTAAATCTTGG CCGTTCTTGTCGATGAAGGT
LITAF	TATAACGCCGGTGCTCTGAC AGAAGGGGATGAAGCAGCAG
CITED2	GTTTGGACCGCATCAAGG CTGCTGTTTGCACACGAAGT
HSPA6	CGCAAAATGCAAGACAAGTG CTTGAGTGCCACAACTGCTG

Gene	Primer sequence (5'– 3')
ST13	CTGGAATGCCTGGACTCAAT TCCTGGAAAGCCACCATAAC
HSPD1	TGCTATGGCTGGAGATTTTG CAGCAGCATCCAATAAAGCA
CASP-1	TGTTCCTGTGATGTGGAGGA CATCTGGCTGCTCAAATGAA
CCNE2	GAAGATTCCTATGGAAGACAGACA CCCCCTTTTCTGAAGGTGTT
CASP4	TGCTGCCACCTAGAGGAAGT GTCGTTCTATGGTGGGCATT
CD44	TTTGCATTGCAGTCAACAGTC CTTGGCTTTCTGTCCTCCAC
TGFB1	CCTACATTTGGAGCCTGGAC CCGGGTTATGCTGGTTGTA
CDC25A	CCCAAACTCCACTACCCTGA GGTAGCTAGGGGGCTCACAG
DNAJB1	GATGGCTCTGATGTCATTTATCC AGAGTGGGGACGTTCACTGT
DNAJB4	ACATTAATTGAGCAGCCACAC TCCAAAATTTAAAAGGGATGGA
DNAJA1	AAGGGTCGCCTAATCATCG GGGTAGGAGTTTTTCCAGCA
DNAJC7	CGCTATGACAGTGGACAGGA GCCAAAGAATGCCTTGAAGA
HSPA1B	CCGAGAAGGACGAGTTTGAG CTGGTACAGTCCGCTGATGA
HSPB1	GGACGAGCATGGCTACATCT GGACAGGGAGGAGGAAACTT

Supplementary Table 4 List of the primers used for real time QPCR.

Supplementary Materials and Methods

Real time QPCR

RNA was extracted from PU-H71-treated and control cells using RNeasy Plus kit (Qiagen) following the manufacturer instructions. cDNA was synthesized using High Capacity RNA-to-cDNA kit (Applied Biosystems). We amplified specific genes with the primers listed in the **Supplementary Table 4** using the Fast SYBR Green conditions (initial step of 20 sec at 95 °C followed by 40 cycles of 1 sec at 95 °C and 20 sec at 60 °C). The C_T values of the housekeeping genes (*GADPH, HPRT* and *B2M*) were averaged and subtracted from the correspondent genes of interest (ΔC_T). The standard deviation of the difference was calculated from the standard deviation of the C_T values (triplicates). Then, the ΔC_T values of the PU-H71-treated cells were expressed relative to their respective control-treated cells using the $\Delta\Delta C_T$ method. The folds of expression for each gene in cells treated with the drug relative to control treated cells is determined by the expression: $2^{-\Delta\Delta CT}$. Results were represented as fold expression with the standard error of the mean for replicates.

Western Blotting

Lysates from DLBCL cells and tumor tissues were prepared using 50 mM Tris pH 7.4, 150 mM NaCl and 1% NP-40 lysis buffer. Lysates for nuclear and cytoplasmatic fractions were obtained using a fractionation kit (Biovision) following the manufacturer's instructions. Protein concentrations were determined using the BCA kit (Pierce). Fifty micrograms of protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the indicated specific primary antibodies: rabbit to Hsp90-α (Assay Designs), mouse to Hsp90-β (Zymed), mouse to Hsp90-α/β (Assay Designs), goat to Hsp90-α/β (Santa Cruz), mouse to Hsp70 (Assay Designs), rabbit to Akt (Millipore), rabbit to c-Raf (Santa Cruz), rabbit to p85-PARP (Promega), rabbit to PARP1 (p116/p85) (E78) (Novus Biologicals), rabbit to IKKγ (Santa Cruz), rabbit to Bcl6 N3 (Santa Cruz), rabbit to Bcl6 C19 (Santa Cruz), mouse to Bcl6 D8 (Santa Cruz), rabbit to GAPDH (Santa Cruz), rabbit to Actin (Santa Cruz), rabbit to Histone3 (Abcam), rabbit to AUF1 (Millipore), rabbit to HA (Sigma), and rabbit to Hsp27 (Assay Designs). Membranes were then incubated with a peroxidase-conjugated correspondent secondary antibody. Detection was performed using an ECL detection system (Vector) according to the manufacturer's instructions. Densitometry values were obtained by using the ImageJ 1.40g software (NIH).

Protein decay determination

Cells growing in complete medium were treated with Cycloheximide (Sigma) 5 μM for 5 minutes to inhibit translation, and then with PU-H71 0.5 μM or vehicle for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h and 24 h. Cells were harvested at each time point and baseline (Cycloheximide

alone) and immediately lysed and processed for western blotting as described above.

mRNA decay determination

Cells were treated with PU-H71 0.5 μM or control for 2 h, and then with Actinomycin-D (Sigma) at 5 μg ml^{-1} to inhibit transcription for 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. Time courses were limited to six hours to avoid affecting cellular mRNA decay by Actinomycin-D enhanced cell death. Cells were harvested at each time point and baseline, the mRNA was stabilized in RNAprotect (Qiagen), purified with the RNAeasy Plus kit (Qiagen), converted to cDNA and quantified by real time QPCR as described above. Data were analyzed by nonlinear regression and half-life was calculated from the first-order decay constant as described[1].

Immunoprecipitations and PU-H71-bead precipitations

Briefly, nuclear extracts were obtained by incubating cells in a buffer containing 10 mM Hepes-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF and protease inhibitor cocktail (Roche) to obtain the cytosolic fraction, followed by incubation of the pellet in a buffer containing 20 mM Hepes-KOH pH 7.9, 25% glycerol, 400 mM NaCl, 1.5 mM $MgCl_2$, 20 mM Na Molybdate, 0.5 mM PMSF and protease inhibitor cocktail (Roche) to obtain the nuclear fraction as supernatant. The nuclear fraction was pre-cleared with protein G beads (Roche) and incubated with no antibody (input), mouse to Hsp90 (Zymed), rabbit to Bcl6 N3 (Santa Cruz) and mix of rabbit and mouse IgG (Zymed). Protein-antibody complexes were precipitated using protein G beads and immunobloted with goat antibody to Hsp90 (Santa Cruz) and mouse antibody to Bcl6 D8 (Santa Cruz). PU-H71-bead and control-bead precipitations were done as described[2] with minor modifications. Incubation and washing buffer contained 20 mM Hepes-KOH pH 7.3, 50 mM $MgCl_2$, 20 mM Na Molybdate and 0.01% NP-40.

Chromatin Immunoprecipitation (ChIP)

ChIP was made as previously described[3,4] with modifications, briefly 10^8 cells were fixed with 1% formaldehyde, lysated and sonicated (Branson sonicator, Branson). Bcl6 N-3 (Santa Cruz) and Hsp90 (Zymed) antibodies were added to the pre-cleared sample and incubated overnight at 4 °C. Then, protein-A or G beads were added, and the sample was eluted from the beads followed by de-crosslinking. The DNA was purified using PCR purification columns (Qiagen). Quantification of the ChIP products was performed by quantitative PCR (Applied Biosystems 7900HT) using Fast SYBR Green (Applied Biosystems). Primers designed for Bcl6 binding sites were previously described[4].

Plasmid constructs

The eGFP-BCL6-HA expression vector was constructed by PCR amplification of the eGFP-

human *BCL6* cDNA sequence from FUW-hBCL6[5] and incorporation of a C-terminal hemagglutinin (HA) tag, which was cloned into the *BamH*I/*Not*I sites of pcDNA3.1 (Invitrogen). The eGFP-BCL6$^{\Delta PEST}$-HA expression vector was also constructed in pcDNA3.1 but contains a deletion of the human BCL6 PEST domain (amino acids 300–417[5]). The eGFP empty vector contains the eGFP cDNA sequence alone from FUW-hBCL6 in pcDNA3.1. Sequence analysis was used to confirm the integrity of all constructs.

Transient transfection of NIH-3T3 cells and DLBCLs

The eGFP, eGFP-BCL6-HA and eGFP-BCL6$^{\Delta PEST}$-HA plasmids were transfected into NIH-3T3 cells using Lipofectamine 2000 (Invitrogen) and into DLBCL cell lines by electroporation (Amaxa nucleofector, Amaxa). Cells were recovered for 24 h prior to harvest. Immunoblots using an anti-HA antibody (Sigma) was used to confirm the expression of exogenous Bcl6 and BCL6$^{\Delta PEST}$ proteins in transfected cells. Cells were transfected with 5 mg of plasmid and sorted for GFP expression on a MoFlo Cell Sorter (Beckman Coulter). Cells were resuspended in RPMI complete medium and treated with vehicle or PU-H71 for 24 h.

In vitro Bcl6/Hsp90 pull down assay

PU-H71-beads and control-beads were incubated overnight with full length Hsp90 expressed in vitro using a pGEM4Z Hsp90 vector[6] in the presence of S^{35} labeled full-length Bcl6, Bcl6 BTB-domain, Bcl6 RD2 middle region and Bcl6 zinc fingers. Bcl6 full-length and fragments were expressed in vitro from T7plink vectors[7]. In vitro expression of proteins was carried out using a T_NT Quick coupled transcription/translation reticulocyte system (Promega) according to the manufacturer's instructions. Sequence analysis was used to confirm the integrity of all constructs.

Apoptosis/necrosis determination

Morphological features of cell death were explored by observing the differential uptake of acridine orange (Sigma) and the DNA binding dye ethidium bromide (Sigma) (AO/EB)[8]. After incubation with PU-H71 or vehicle, cells were gently centrifuged at 4 °C and resuspended in 20 μl of PBS plus 2 μl of a 1:1 mix of 100 μg/ml of AO/EB. Stained cell suspension (10 μl) were transferred to a microscope slide and viewed under a fluorescent light microscope (Axioskop 2, Carl Zeiss AG) with 100, 200 and 400X magnifications. A total of 300 cells were counted in triplicates per treatment condition.

Caspase 7 and 3 activity

The activity of caspase-7 and caspase-3 was determined using the Apo-ONE caspase 3/7 assay (Promega)[8]. Cell lines were treated with PU-H71 or control for 12 h followed by 1 h exposure to the pro-fluorescent Z-DEVD-R110 substrate. Activation of Z-DEVD-R110 by the activity of

caspases 3 and 7 allows the R110 group to become intensely fluorescent (Ex_{499nm} / Em_{521nm}), which was measured using the Synergy4 microplate reader in four replicates. Caspase 3 and 7 activity was related to the cell number determined by CellTiter-Blue (Promega) in a multiplex assay.

BCL6 small-interfering RNA

Predesigned siRNA targeting BCL6 (a equimolar mix of Invitrogen 12923D06 and 12923D07) and control (a sequence with no significant similarity to any vertebrate gene, Invitrogen 12935-110 Lo GC Duplex 2) were obtained from Invitrogen. Cells were transfected with 2 μM of siRNA by electroporation (Amaxa electroporator) using the transfection buffer recommended by the manufacturer (Amaxa) or subjected to the procedure without siRNA (mock control).

Pharmacokinetic (PK) study

For PK determinations, tumors were allowed to reach 1 cm in diameter before treatment. Mice bearing Farage, OCI-Ly7 and Toledo tumors were administered PU-H71 75 mg per kg body weight by intra-peritoneal injection. Two animals per time point and two controls were sacrificed by cervical dislocation under anesthesia at 6, 12 and 24 h after the administration of PU-H71. At sacrifice tumor from all mice and plasma, kidney, liver, heart, lung, spleen and small intestine tissues were collected from Farage mice. A gross necropsy was performed on all mice. For quantitative HPLC analysis, tumor and normal tissue samples were homogenized in ethanol:water (2:1) solution at a 1:3 weight:volume ratio. Concentrations of PU-H71 were determined by high-performance liquid chromatography-mass spectrometry (HPLC-MS) at the Analytical Pharmacology Core Facility of the Sloan-Kettering Institute. Results are expressed as μg of PU-H71 per gram of tissue and normalized to control (time 0).

Mice toxicity studies

The Animal Institute Committee of the Albert Einstein College of Medicine approved all animal procedures. In addition to the toxicity studies in the xenografted SCID mice, we conducted specific toxicity studies in Balb/c mice. Six to eight-week old male Balb/c mice were purchased from the US National Cancer Institute and housed in a clean environment. Mice (n = 15) were randomized in three groups of 5 and intra-peritoneally injected for 10 consecutive days with vehicle (0.2 ml per day), PU-H71 50 mg per kg body weight per day or PU-H71 75 mg per kg body weight per day. Mice were weighed and examined every other day. Mice were euthanized by cervical dislocation under anesthesia at indicated times when blood and tissues were harvested and microscopically examined.

Human β2-microglobulin

Serum levels of human β2-microglobulin were determined in the mice at day 10 by enzyme immunoassay (Quatikine IVD, R&D Systems). The mean absorbance (A_{450} – A_{620}) values for each set of triplicates and standards were measured using the Polarstar Optima microplate reader (BMG Labtechnologies) and concentrations were calculated using a four-parameter logistic curve fit (SigmaPlot, Systat Software).

Apoptotic index

DNA fragmentation coupled to the apoptotic response was detected in morphologically identifiable nuclei and apoptotic bodies present in formalin-fixed paraffin-embedded tumors by the TUNEL assay (ApopTag, Chemicon) following the manufacturer instructions with optimization. Tissue slides were pre-treated with 0.5% trypsin for 15 minutes (Zymed), to improve the exposure of DNA.

Gene expression microarrays

Farage tumors were harvested from 6 mice and maintained in Allprotect (Qiagen) at -80 °C until RNA isolation using RNeasy Plus kit (Qiagen). RNA integrity was determined using the RNA 6000 Nano LabChip Kit on Agilent 2100 Bioanalyzer (Agilent Technologies). Two independent samples were analyzed at 0 (control), 6 and 12 h after the administration of PU-H71 75 mg per kg body weight intra-peritoneally. RNA (6 µg) was hybridized to GeneChip Human Genome U133 Plus 2.0 Expression arrays (Affymetrix) according to the manufacturer's recommendations. After hybridization, the chips were processed using fluidics station 450, high-resolution microarray scanner 3000, and GeneChip Operating Software workstation version 1.3. The .cel files were imported into BRB-ArrayTools software (Biometric Research Branch, NCI) by setting up robust multi-array analysis background correction. Probe sets showing minimal variation across arrays were excluded by selecting only those whose expression differed by at least 1.5 folds from the median of the arrays in at least 1 array. This filter yielded 848 probe sets, which were further filtered to remove those that did not show at least a 2-fold expression change from baseline (time 0) in at least one time point after the administration of PU-H71 (times 6 h and 12 h). Additional probe sets lacking GeneID information were excluded from further analysis. Lists of unique up-regulated (79) or down-regulated (204) genes were obtained and further uploaded into the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov). The list of up- and down-regulated genes that constitutes the PU-H71 gene expression signature was compared to that of a collection of drug-treated cell lines, the Connectivity Map version 1.0 (http://www.broad.mit.edu/cmap/). The PU-H71-associated gene list was also analyzed for ARE containing genes by using the ARED Integrated search engine (http://brp.kfshrc.edu.sa/AredInteg/). Expression profiling data have been deposited in NCBI's

Gene Expression Omnibus and are accessible through GEO series accession number GSE13401 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13401).

Tissue microarray (TMA)

The TMA was made using paraffin-embedded tissues obtained from the files of the Department of Pathology of the New York Presbyterian Hospital in accordance with the Institutional Review Board guidelines. This set included 70 cases of DLBCLs (classified in accordance with World Health Organization classification system) and 20 controls, and was analyzed for Hsp90-α, Hsp90-β, Hsp90-α/β expression in the nucleus and cytoplasm of tumor cells. Bcl6 staining was previously reported[8]. The TMAs were made using Beecher Instruments microarrayer (Silver Spring, MD). All of the cores contained malignant cells, and no discrepant results were seen between duplicate cores. We identified antigens of interest as previously described[8]. Briefly, deparaffinized slides were antigen retrieved in citrate buffer pH 6.0 (Zymed) then first antibody was applied, followed by incubation with a corresponding biotinylated secondary antibody (Vector Laboratories). We incubated slides with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex, Vectastain ABC (Vector). Color was developed with diaminobenzoate chromogen peroxidase substrate (Vector). The antibodies used were specific for Hsp90-α (Array Designs), Hsp90-β (Zymed) and Hsp90-α/β (Santa Cruz). Two independent researchers scored the TMA results.

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