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Supplemental Material to:

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Inhibitors of SRC kinases impair antitumor activity of anti-CD20 monoclonal antibodies

mAbs 2014; 6(5)
http://dx.doi.org/10.4161/mabs.32106

http://www.landesbioscience.com/journals/mabs/article/32106

SUPPLEMENTARY INFORMATION

Supplementary Table 1. Influence of dasatinib on surface CD20 expression and survival in primary cells.

No	Type of tumor	Effects of dasatinib on the surface CD20 levels (MFI - % of controls)			Effects of dasatinib on the surface CD20 levels (MFI)				Survival (% of control)		
		20 nM	100 nM	200 nM	CON	20 nM	100 nM	200 nM	20 nM	100 nM	200 nM
Pt1	CLL	75.6	48.2	48.5	39	29.5	18.8	18.9	92.1	87.6	88.8
Pt2	CLL	44.5	38.9	36.4	290	129.05	112.8	105.6	82.9	73.2	62.2
Pt3	CLL	67.7	47.4	48.2	280	189.6	132.7	134.9	60	53.3	53.3
Pt4	CLL	56.1	42.2	40.3	70	39.3	29.5	28.2	74	57.1	57.1
Pt5	CLL	75.1	71.2	67.4	65	48.8	46.3	43.8	66.7	54.7	48
Pt6	CLL	77.9	76.4	72.9	122	95	93.2	88.9	94.3	100	97.1
Pt7	CLL	90.2	92.1	39.0	38	34.3	35	14.8	105.7	105.7	108.6
Pt8	CLL	58.2	70.9	56.5	251	146.1	178	141.8	81.8	69.7	65.1
Pt9	CLL	79.4	77.9	71.2	126	100	98.1	89.7	95.6	88.4	84
Pt10	CLL	53.2	52.2	49.0	66	35.1	34.4	32.3	95	88.3	88.3
Pt11	CLL	58.8	61.2	59.4	46	27	28.1	27.3	89.9	87.3	84.8
Pt12	CLL	67.1	70.8	71.3	39	26.2	27.6	27.8	85.2	66.7	61.1
Pt13	CLL	45.3	42.3	42.2	91	41.2	38.5	38.4	79.4	75	67.6
Pt14	CLL	46.4	54.5	55.7	667	309.5	363.5	371.5	90.1	87.3	84.5
Pt15	CLL	75.2	66.7	68.9	76	57.1	50.7	52.4	98.3	89.8	101.7
Pt16	CLL	69.7	64.7	65.6	150	104.5	97.1	98.4	81.8	75.8	75.8

Pt17	CLL	68.8	71.5	70.2	69	47.5	49.3	48.4	94	89.5	95.5
Pt18	CLL	45.4	39.6	40.7	93	42.2	36.8	37.8	81	74.7	75.9
Pt19	CLL	74.5	78.0	77.6	49	36.5	38.2	38	87.5	89	82.8
Pt20	CLL	81.8	91.1	92.2	61	49.9	55.6	56.2	95.8	94.4	95.8
Pt21	CLL	97.0	83.9	84.3	42	40.7	35.2	35.4	100	92.4	100
Pt22	CLL	102.1	90.6	85.8	129	131.7	116.9	110.7	101.9	100	105.7
Pt23	CLL	99.6	99.9	105.8	103	102.6	102.9	109	94.5	91.8	105.5
Pt24	CLL	115.3	108.3	81.4	26	30	28.2	21.1	78.9	78.9	105.3
Pt25	CLL	80.4	86.6	89.5	78	62.7	67.5	69.8	101.8	98.2	100
Pt26	FL	58.3	55.0	52.8	250	145.8	137.5	132	83.3	79.2	66.7
Pt27	MCL	74.8	54.8	51.3	133	99.5	72.9	68.2	69.1	58.2	54.5
Pt28	MCL	59.1	53.2	51.8	80	47.3	42.6	41.4	52.8	58.3	47.2
Pt29	MCL	81.5	77.2	79.5	56	45.6	43.2	44.5	94.8	94.8	94.8
Pt30	MCL	88.3	81.9	83.9	99	87.4	81.1	83.1	81.8	78.2	56.4

Supplementary Table 2. Changes in the expression of genes of interest in Raji cells incubated for 24 h with dasatinib or PP2.

Protein name	**Gene name**	**Log FC**	***p*-value**
CD19	*CD19*	-0,548	*0,12805*
CD20	***MS4A1***	**-2,638**	*0,0011*
CD21	*CR2*	-1,07	*0,01232*
CD21	*CR2*	-1,37	*0,00001*
CD22	***CD22***	**-2,304**	*0,00003*
CD38	*CD38*	0,5	*0,00177*
CD46	*CD46*	-0,104	*0,14952*
CD52	*CD52*	0,065	*0,73226*
CD55	*CD55*	-0,112	*0,41429*
HLA-DR	*HLA-DRA*	-0,124	*0,30384*
HLA-DR	*HLA-DRB1*	-0,525	*0,03945*
HLA-DR	*HLA-DRB3*	-0,403	*0,00401*
HLA-DR	*HLA-DRB4*	-0,467	*0,00735*
HLA-DR	*HLA-DRB5*	-0,452	*0,06672*
HLA-DR	*HLA-DRB6*	-0,628	*0,00881*
ICAM1	*ICAM1*	-0,71	*0,0688*

The expression level of genes encoding proteins of interest (same as in Supplementary Fig. S6) was extracted from the microarray data and analyzed as in Fig. 1A. All values of log fold change (Log FC) below -1.584 (fold change decrease over 3.0) were marked in bold.

METHODS

Cell culture

Supplementary Table 3. Cell lines - culture conditions and source.

Medium	Cell lines
RPMI-1640 (Gibco) + 10% FBS	Raji (ATCC), Ramos (ATCC), Daudi (ATCC), HS-Sultan (ATCC), EHEB (ATCC), MEC-1 (ATCC), DoHH2 (DSMZ), SU-DHL-4 (ATCC), SU-DHL-6 (ATCC), Karpas-422 (DSMZ), Pfeiffer (ATCC), Toledo (ATCC), NALM-6 (DSMZ), HEK-293T (ATCC), K562 (ATCC), EL-4 hCD20 (kindly provided by JHW Leusen)
IMDM (Gibco) + 10% FBS	Ly-1, Ly-4, Ly-7, Ly-10, Ly-19 (all kindly provided by Prof. M.A. Shipp), primary cells
DMEM (Sigma Aldrich) + 10% FBS	HeLa (ATCC)

All media were supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B (Sigma Aldrich). All the cells upon receipt from the original source were not passaged over 6 months in our laboratory.

Gene expression microarray

RNA samples were analyzed using BioAnalyzer (Agilent. USA) to estimate the final RNA quality and integrity. Only samples with *RNA integration number (RIN)*=10 were further taken for the analysis. The Quick Amp Labeling Kit (Agilent) was used to amplify and label target RNA and to generate complementary RNA (cRNA). The hybridization was performed with human-specific AMADID Release GE 8×60K microarrays (Agilent) using Gene Expression Hybridization Kit (Agilent) according to the manufacturer`s protocol. Each sample was examined in two dye-swap repetitions to eliminate the effect of label factor. Thus, for each biological condition (cells incubated with 100 nM dasatinib or 10 µM PP2 and control cells) the sample was labeled twice with Cy3 and twice with Cy5. Acquisition and analysis of hybridization intensities were carried out using DNA microarray scanner (Agilent, Santa Clara, CA, USA). The Gene Spring (Agilent) hierarchical clustering depicted similar gene expression in all dye-swap

experiments indicating that all microarray samples were successfully labeled. hybridized. scanned and that are highly reproducible.

NK cell functional and cytotoxicity assays

NK effector cells were isolated from PBMC of healthy donors with magnetic EasySep® Human CD56 Positive Selection Kit (STEMCELL Technologies). ADCC was performed in the presence of dasatinib or PP2 (co-incubation model). CFSE-labeled Raji cells were incubated for 4 h with anti-CD20 mAbs (100 µg/ml) and NK cells at effector to target (E:T) ratio 6:1 and subsequently stained with PI. The percentage of cellular cytotoxicity was calculated in the CFSE-positive population. For functional assays NK cells were negatively isolated from PBMC with EasySep® system (STEMCELL Technologies) and activated with recombinant IL-2 (rIL-2; Proleukin. 100 IU/ml; Chiron) and recombinant IL-15 (10 ng/ml; R&D Systems) overnight. NK cells were incubated for 4h with Raji cells at E:T ratio 1:1 and 100 µg/ml rituximab in the presence of GolgiStop (BD Biosciences), anti-CD107a FITC-conjugated antibody (BD Biosciences) and dasatinib or PP2 (co-incubation model). NK cells were stained with PE-Vio770-conjugated anti-CD56 (MACS Miltenyi). PerCP-Cy5.5-conjugated anti-CD3 (BD Biosciences) and Fixable Viability Dye (eBioscience). To determine cytokines production cells were permeabilized with Cytoperm/Cytofix (BD Biosciences) and stained with Alexa Fluor®700-conjugated anti-IFN-γ antibody (BD Biosciences) and eFluor®450-conjugated anti-TNF-α (eBioscience) before cell analysis using BD FACSCanto II (BD Biosciences. La Jolla. CA. USA). Results are presented as a percentage of positive NK cells (TNF-α. IFN-γ. CD107a) within the whole NK-cell population. Spontaneous NK cell cytotoxicity assay was performed using CFSE/PI flow cytometry assay with K562 cells in presence of increasing concentrations of dasatinib or PP2 (co-incubation model). K562 cells fluorescently labeled with CFSE were incubated for 4 h in 96-well U-bottom plate (5×10^5 cells/well) with NK cells at effector to target ratio 6:1 and subsequently stained with PI. The percentage of cellular cytotoxicity was calculated as described before.

Flow cytometry

Supplementary Table 4. Antibodies used for flow cytometry studies.

antibody	fluorochrome	clone	company
IgG1	FITC	X40	Becton Dickinson
anti-CD19	FITC	4G7	Becton Dickinson
anti-CD20	FITC	L27	Becton Dickinson
anti-CD21	FITC	B-ly4	Becton Dickinson
anti-CD22	FITC	S-HCL-1	Becton Dickinson
anti-CD38	FITC	HB-7	Becton Dickinson
anti-CD45	FITC	2D-1	Becton Dickinson
anti-CD45RA	FITC	L48	Becton Dickinson
anti-CD46	FITC	E4.3	BD Pharmingen
anti-CD55	FITC	IA10	BD Pharmingen
anti–HLA-DR	FITC	L243	Becton Dickinson
anti-CD20	PE	2H7	Becton Dickinson

Real-time PCR

RNA from Raji cells with silenced expression of FYN. LCK or LYN or transduced with non-silencing pGIPZ or pLVTHm was used for the first-strand cDNA synthesis and primed with oligo(dT) using AMV-reverse transcriptase (EURx). Real-time PCR was performed using LightCycler® Fast Start DNA Master PLUS SYBRGreen I (Roche) as described before. The following primers were used (Supplementary Tables 4-6):

Supplementary Table 5. Primers sequences and annealing conditions.

	Forward primer	Reverse primer	Annealing temperature
β$_2$m	TAGGAGGGCTGGCAACTTAG	CCAAGATGTTGATGTTGGATAAGA	60°C
RPL29	CAGCTCAGGCTCCCAAAC	GCACCAGTCCTTCTGTCCTC	60°C
β-actin	TTCCTTCCTGGGCATGGAGT	ATCCACATCTGCTGGAGGGT	60°C
CD20	GAATGGGCTCTTCCACATTGCC	TCTCCGTTGCTGCCAGGAGT	60°C
FYN	CCCCAACTACAACAACTTCC	CTCCTGTTCCTCCTCTCGTA	57°C
LCK	CTGCCATTATCCCATAGTCC	GATTGGAGCCTTCGTAGGTA	57°C
LYN	GTATCCAGTGGTGTGCAAAA	AGTCAGAATCTTGGCGGTAG	57°C

Supplementary Table 6. Primers sequences and annealing conditions.

	Forward primer	Reverse primer	Annealing temperature
β$_2$m	TAGGAGGGCTGGCAACTTAG	CCAAGATGTTGATGTTGGATAAGA	60°C

RPL29	CAGCTCAGGCTCCCAAAC	GCACCAGTCCTTCTGTCCTC	60°C
RPL30	#7014 (Cell Signaling)	#7014 (Cell Signaling)	60°C
β-actin	TTCCTTCCTGGGCATGGAGT	ATCCACATCTGCTGGAGGGT	60°C
CD20	GAATGGGCTCTTCCACATTGCC	TCTCCGTTGCTGCCAGGAGT	60°C

Supplementary Table 7. Primers and probes sequences. annealing conditions.

	Forward primer	Reverse primer	Hydrolysis Probe (5'-to-3'). concentration	Annealing temperature
β_2m	ACCTTCTACAATGAG CTGCG	CCTGGATAGCAACGTA CATGG	FAM-CTAAGGCCACGGAGCGAGACATC-Dabcyl. 100nM	54°C
CD20	GAATGGGCTCTTCCA CATTGCC	TCTCCGTTGCTGCCAG GAGT	FAM-CATACAATCTCTGTTCTTGG-Dabcyl. 250nM	54°C

Plasmids

To generate the luciferase reporter construct with a mutated *CD20* promoter (BAT box mut. Thevenin et al. 1993) the core sequence of the BAT box region. TAAT was exchanged with CCTC sequence. using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. *CD20* coding sequence was amplified by PCR using cDNA from Raji cells and cloned into pLVX-IRES-PURO vector (Clontech) with XhoI and BamHI restriction enzymes or into pTriex-2 Neo vector (Novagen) containing His-tag sequence. The CD20-Histag sequence was then amplified by PCR and cloned into pLVX-IRES-PURO vector using XhoI and BamHI restriction enzymes.

Supplementary Table 8. Truncated *CD20* promoters.

symbol	Lacking binding sites	Promoter region
Del3	NF-κB	-313/+52 bp
Del2	NF-κB. BAT box (binding site for Oct-1 and Oct-2)	-198/+52 bp
Del1	NF-κB. BAT box. PU box (binding site for PU.1/Pip)	-126/+52 bp
Del0	NF-κB. BAT box. PU box. E-box (binding site for TFE3)	-39/+52 bp

Knock-down of FYN, LYN and LCK kinases.

To knock-down FYN and LCK kinases in Raji cells GIPZ lentiviral shRNAmir (Open Biosystems. represented by Thermo Fisher Scientific. Huntsville. AL) were used for the transfection of HEK-293T cells and

lentivirus production. For the knock-down of LYN kinase a pLVTHm lentiviral plasmid backbone was used and the DNA fragment encoding LYN-targeting shRNA was cloned (Supplementary Table 8) with ClaI and MluI restriction enzymes.

Supplementary Table 9. Lyn-targeting sequences

	sequence (5'-to-3')
sense	cgcgtCCCCGTATCAGCGACATGATTAAACTTCAAGAGAGTTTAATCATGTCGCTGATACTTTTTGGAAat
antisense	cgatTTCCAAAAAGTATCAGCGACATGATTAAACTCTCTTGAAGTTTAATCATGTCGCTGATACGGGGa

Lentiviral/retroviral transduction of target cells

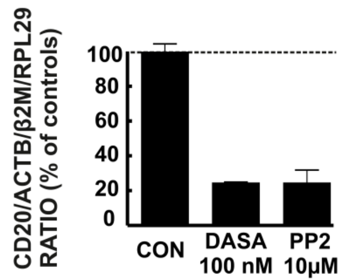
For lentiviruses production HEK-293T cells. seeded into 6-well plates were co-transfected with 2 µg of gene of interest (GOI)-containing vector and components of 2nd generation of packaging vectors, namely 1.5 µg of psPAX2 packaging vector and 1 µg of pMD2.G envelope vector using GeneJuice® transfection reagent (EMD Chemicals Inc.) according to the manufacturer's protocol. 72 h post transfection the lentiviruses-containing medium was collected and added to the culture of target Raji cells at the 2:1 ratio (lentiviruses-containing medium : culture medium). For retroviruses production HEK-293T cells. seeded into 100 mm dish were co-transfected with 10 µg of GOI-containing vector and components of retroviral packaging vectors, namely 10 µg of pKAT packaging vector and 5 µg of pVSV-G envelope vector using Lipofectamine® 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. 24 h post transfection the retroviruses-containing medium was collected, added to Raji cells and the mixture was centrifuged at 460×*g* for 1 h at room temperature. After centrifugation cells were resuspended and cultured with viruses for subsequent 24 h. To enable virus entry to the target cells, 2 µg/ml of polybrene (Sigma) was added to the transduction mixture.

Isolation of B cells

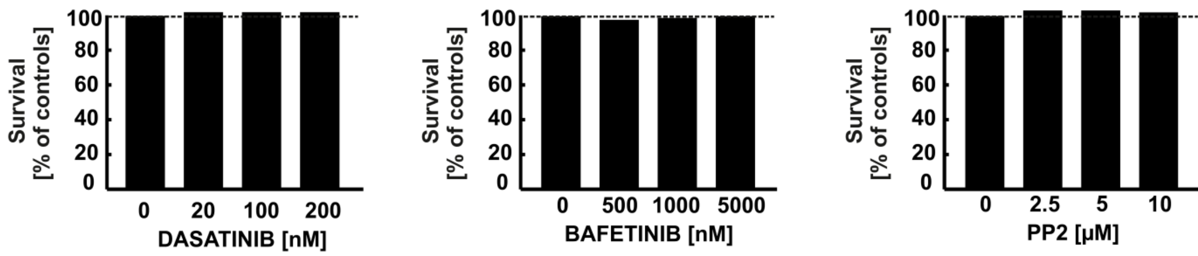
B cells were isolated from peripheral blood mononuclear cells of healthy donors with magnetic EasySep®

Human CD19 Positive Selection Kit (STEMCELL Technologies).

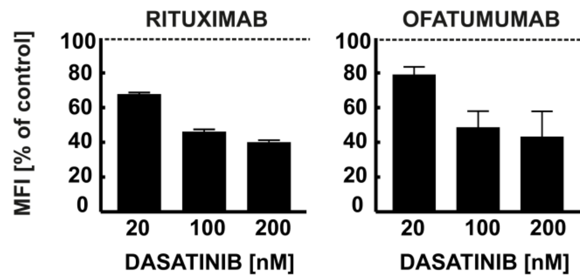
SUPPLEMENTARY FIGURES



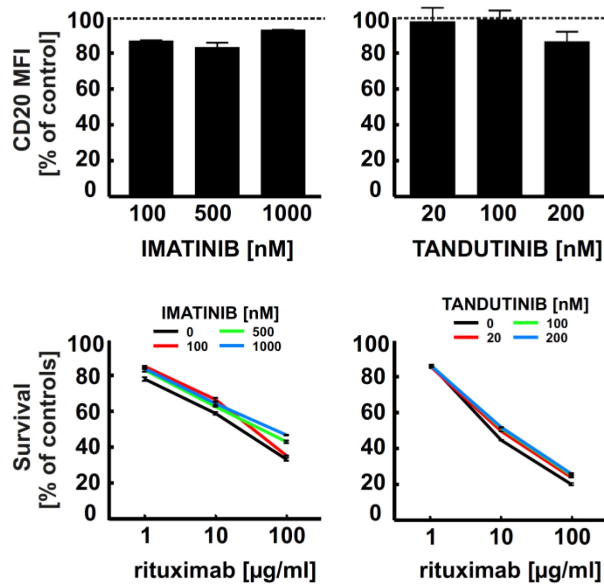
Supplementary Figure S1. Effects of dasatinib and PP2 on CD20 mRNA levels in Raji cells.
cDNA from Raji cells incubated for 24 h with 100 nM dasatinib or 10 μM PP2 was used for qRT-PCR amplification (with LightCycler® Fast Start DNA Master PLUS SYBRGreen I) of CD20, ACTB, β2m and RPL29 products.



Supplementary Figure S2. Survival of Raji cells after 48h incubation with selected Src inhibitors.
Raji cells pre-incubated for 48 h with increasing concentrations of dasatinib, bafetinib or PP2 were subsequently stained with PI (final concentration 4 µg/ml) and analyzed for survival with flow cytometry. Shown is one representative of at least 3 independent experiments.

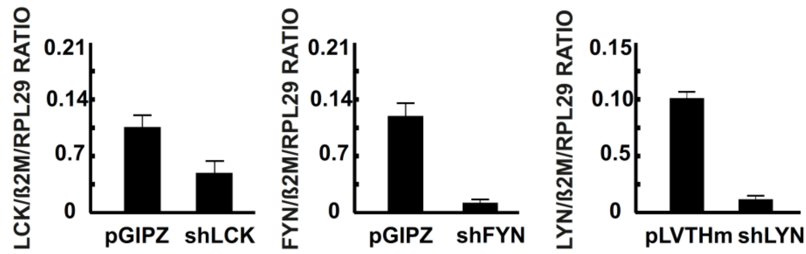


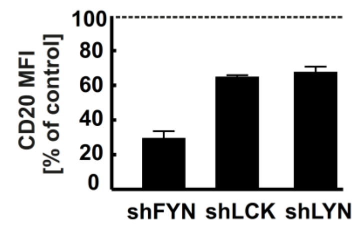
Supplementary Figure S3. Effects of dasatinib on binding of clinically available anti-CD20 mAbs.
Raji cells incubated with increasing concentrations of dasatinib for 48 h were further treated with 100 µg/ml rituximab or ofatumumab followed by incubation with saturating amounts of Alexa Fluor® 488-conjugated secondary antibody. Results are presented as % of controls MFI (± SD) of PI-negative cells. Shown is one representative of at least 3 independent experiments.



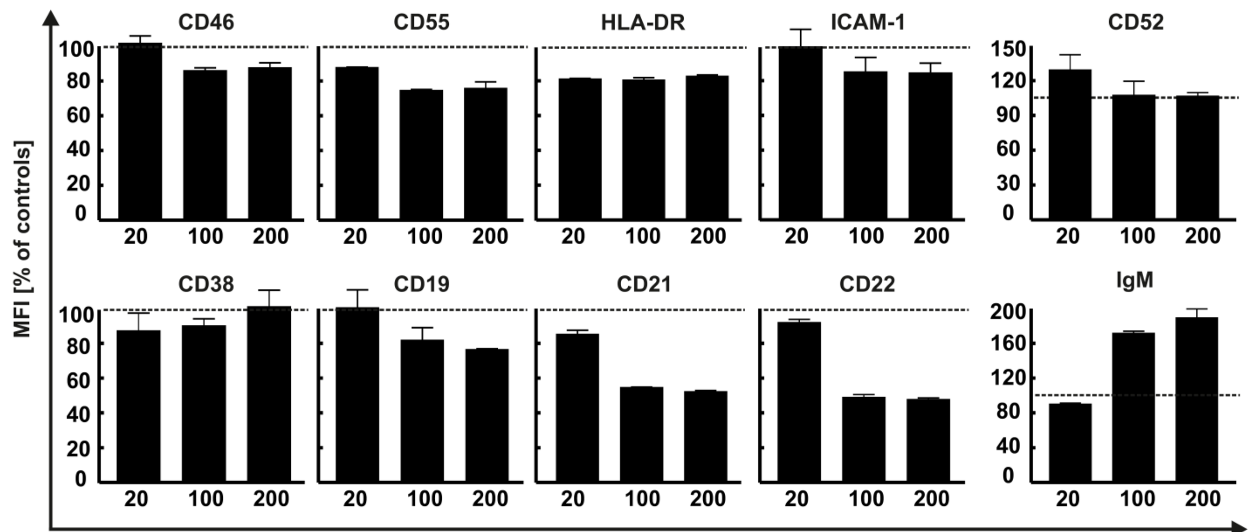
Supplementary Figure S4. Effects of imatinib and tandutinib on the surface CD20 levels and R-CDC/O-CDC.

Raji cells were incubated for 48 h with increasing concentrations of imatinib (Bcr-Abl kinase inhibitor) or tandutinib (type III receptor tyrosine kinases inhibitor) at indicated concentrations. CD20 staining and R-CDC were done as described in the manuscript. Shown is one representative of at least 3 independent experiments.

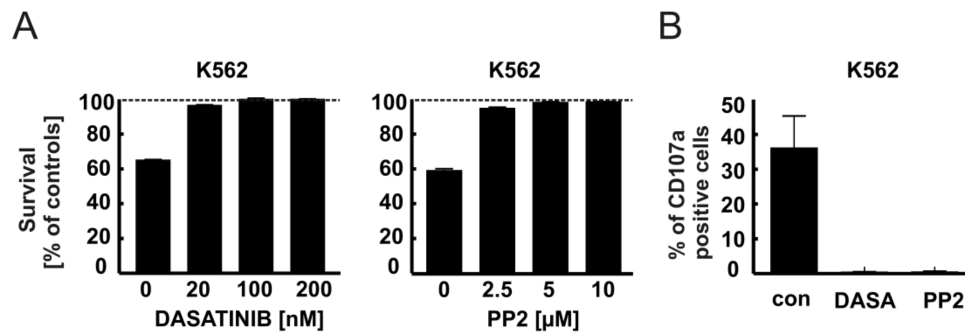




Supplementary Figure S5. Effects of knock-down of FYN. LCK or LYN kinase on surface CD20 expression. (A) cDNA from Raji cells with silenced expression of FYN, LCK or LYN or transduced with non-silencing pGIPZ or pLVTHm was used for qRT-PCR amplification (with LightCycler® Fast Start DNA Master PLUS SYBRGreen I) of FYN, LCK or LYN, CD20, β_2m and RPL29 products. (B) Raji cells with silenced expression of FYN, LCK or LYN or transduced with non-silencing pGIPZ or pLVTHm were incubated with saturating amounts of PE-conjugated anti-CD20 mAb (2H7) or IgG1 isotype control for 30 min at room temperature in the dark, washed with PBS and resuspended in PBS. Binding of antibodies was determined with flow cytometry. Results are presented as MFI (± SD) compared with corresponding controls. Shown is one representative of at least 3 independent experiments.

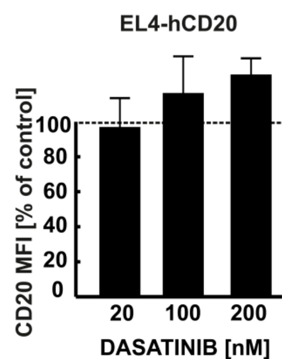


Supplementary Figure S6. Effects of dasatinib on various B-cell surface markers.
Raji cells pre-incubated for 48 h with increasing concentrations of dasatinib were incubated with saturating amounts of FITC-conjugated anti-CD46, anti-CD55, anti-HLA-DR, anti-ICAM-1, anti-CD38, anti-CD19, anti-CD21 or anti-CD22 mAbs for 30 min at room temperature. To determine surface levels of CD52 and BCR the cells were blocked with 1% BSA/PBS for 30 min and incubated for subsequent 30 min with 100 µg/ml alemtuzumab or 10 µg/ml anti-IgM antibody, respectively. After washing the cells were incubated with saturating amounts of Alexa Fluor® 488-conjugated corresponding secondary antibodies and analyzed using flow cytometry. Results are presented as a percentage of MFI of control cells (± SD). Shown is one representative of at least 3 independent experiments.

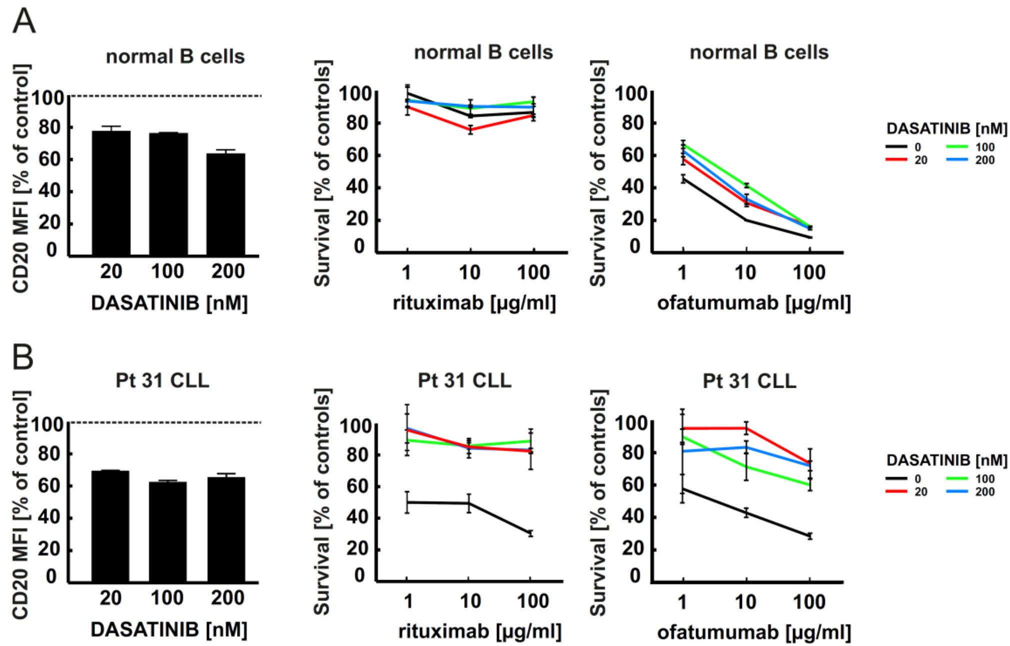


Supplementary Figure S7. Effects of dasatinib and PP2 on NK cells natural cytotoxicity and degranulation in co-incubation model.

(A) K562 target cells (stained with CFSE) were incubated with NK cells for 4 h in the presence of either dasatinib or PP2. K562 cell survival is presented as a percentage of control cells without inhibitors. (B) K562 cells were incubated with NK cells (E:T ratio 1:1) and dasatinib (100 nM) or PP2 (10 µM) for 4 h in the presence of GolgiStop and anti-CD107a antibody. NK cells degranulation was determined using flow cytometry. Results are presented as a percentage of CD107a positive cells (± SD) within the whole NK cell population. Shown is one representative of at least 3 independent experiments.

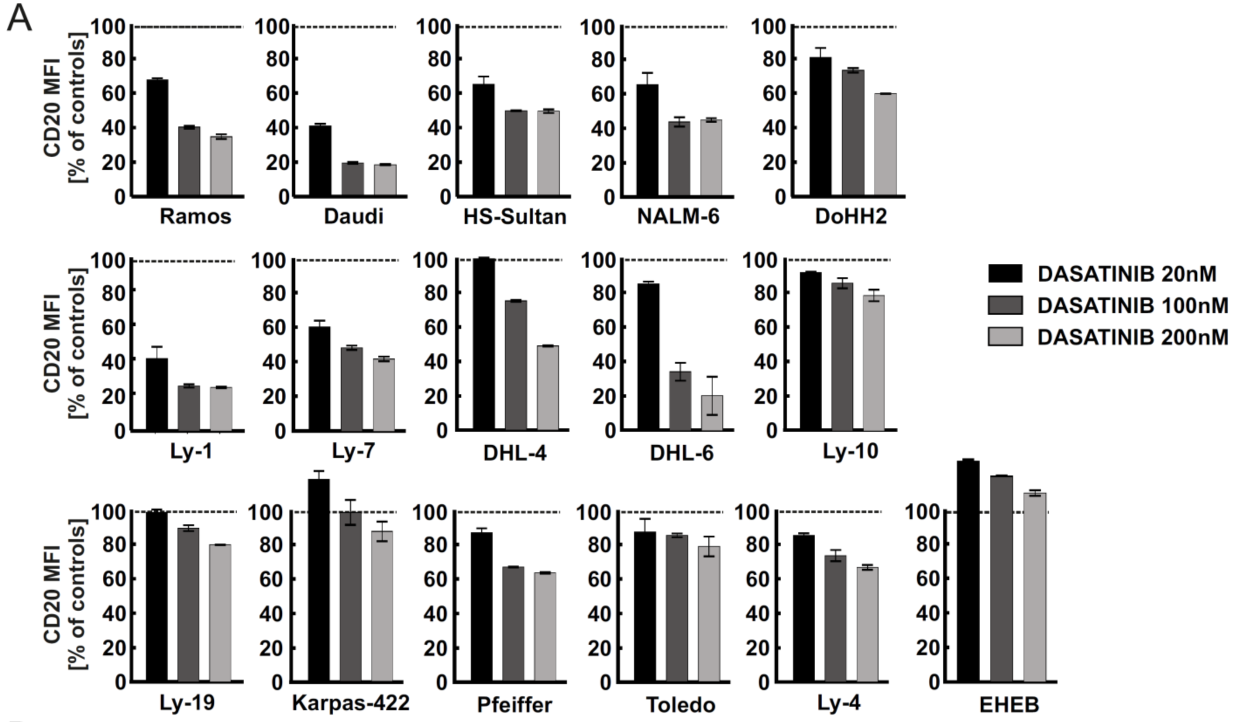


Supplementary Figure S8. The effect of dasatinib on the surface CD20 levels in EL4-hCD20 cells.
The surface CD20 level was analyzed in EL4-hCD20 cells pre-incubated for 48 h with increasing concentrations of dasatinib. Results are presented as a percentage of MFI of control cells. Shown is one representative of at least 3 independent experiments.



Supplementary Figure S9. Effects of dasatinib on the surface CD20 levels and R-CDC/O-CDC in healthy B cells and primary CLL cells.

(A) B-cells freshly isolated from healthy donors were incubated with saturating amounts of FITC-conjugated anti-CD20 mAb (L27) or IgG1 isotype control for 30 min at room temperature in the dark. washed with PBS and resuspended in PBS with 4 μg/ml of PI. Binding of antibodies was determined with flow cytometry. Results are presented as mean MFI (± SD) of PI-negative cells. Cell viability in R-CDC or O-CDC assay was measured with PI assay as described in the manuscript. (B) Primary tumor cells isolated from CLL patient were pre-incubated for 48 h with increasing concentrations of dasatinib. Cell viability in R-CDC or O-CDC assay was measured with PI assay as described in the manuscript.



B

Ramos, Daudi, Sultan, NALM-6, DoHH2

Ly-1, Ly-7, DHL-4, DHL-6, Ly-10

Ly-19, Karpas-422, Pfeiffer, Toledo, Ly-4

Survival [% of control]

rituximab [µg/ml]

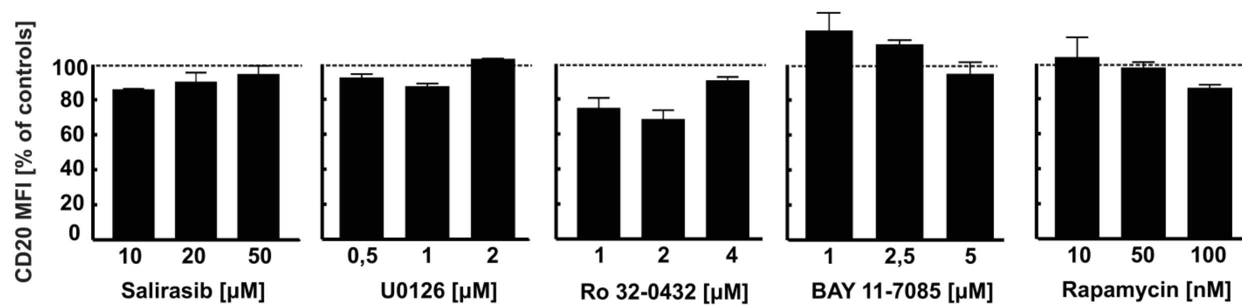
ofatumumab [µg/ml]

DASATINIB [nM]

0, 20, 100, 200

Supplemental Figure S10. Effects of dasatinib on CD20 levels and R-CDC/O-CDC in B-cell tumor cell lines.

(A) Ramos, Daudi, HS-Sultan, NALM-6, DoHH2, Ly-1, Ly-7, DHL-4, DHL-6, Ly-10, Ly-19, Karpas-422, Pfeiffer, Toledo, Ly-4 or EHEB cells pre-incubated for 48 h with increasing concentrations of dasatinib were incubated with saturating amounts of FITC-conjugated anti-CD20 mAb (L27) or IgG1 isotype control for 30 min at room temperature in the dark, washed with PBS and resuspended in PBS with 4 µg/ml of PI. Binding of antibodies was determined with flow cytometry. Results are presented as MFI (± SD) of PI-negative cells. (B) Cell viability in R-CDC/O-CDC (in case of rituximab resistance) assay was measured with PI assay. Cell viability was determined in presence of 10% serum and measured as previously described. The survival of cells is presented as a percentage of control cells without rituximab/ofatumumab. Shown is one representative of at least 3 independent experiments.



Supplementary Figure S11. Effects of selected pathways inhibitors on the surface CD20 levels.

Raji cells pre-incubated for 48 h with increasing concentrations of various inhibitors were analyzed for surface CD20 levels as described in the manuscript. Results are presented as mean MFI (± SD) of PI-negative cells. Shown is one representative of at least 2 independent experiments.