Supplementary Information

Tumor antigen ROR1 targeted drug delivery mediated selective leukemic but not normal B cell cytotoxicity in chronic lymphocytic leukemia

Running Title: ROR1 targeted delivery of OSU-2S in B-CLL

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Supplementary Information Includes

Supplementary Methods:

Immunoblotting:

Cells were lysed with lysis buffer containing 10mM Tris (tris(hydroxymethyl)aminomethane) pH 7.4, 150mM sodium chloride, 1% Triton X-100, 1% deoxycholic acid, 10% SDS and 5mM EDTA. Cytoplasmic and nuclear extracts were prepared with NE-PER extraction kit (Pierce, Thermo scientific, Rockford, IL). Proteins were quantified by bicinchoninic acid (BCA) method (Pierce), separated by SDS-PAGE gel, transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were probed with commercially obtained indicated antibodies, followed by horseradish peroxidase (HRP) conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) and detected by addition of chemiluminescent substrate (Pierce). Quantification was done with Chemi-Doc system with Quantity one software (Bio-Rad Laboratories, Hercules, CA).

Phosphatase assay:

SHP1 and PP2A enzyme activity were measured using DuoSet®IC (R&D Systems, Minneapolis, MN) and PP2A immunoprecipitation phosphatase assay kit (Upstate/Millipore, Billerica, MA) respectively as per manufacturer's instructions. In brief, phosphatase enzymes were immunoprecipitated from CLL cell extracts using anti-SHP1/PP2A antibody conjugated/and agarose beads and incubation of enzyme bound beads with phospho peptide substrate to release free phosphate, followed by detection using malachite green solution and molybdic acid. The phosphatase activity is expressed as amount of free phosphate released from the reaction normalized to the levels of enzyme immunoprecipitated as quantified by immunoblotting to avoid variability in immunoprecipitation across the samples.

Immunoprecipitation:

Immunoprecipitation was carried out by incubating cell lysates with primary antibody and protein A agarose beads (Millipore, Billerica, MA) in Tris buffered saline-tween20 (TBST) overnight at 4°C in a rocker, followed by washing the beads 6 times with ice cold TBST. The beads were boiled with Laemmeli buffer before separation by SDS-PAGE.

Confocal fluorescence microscopy:

Nuclear SHP1^{S591}: Experimental cells were pre stained with membrane labeling dye PKH26 (Sigma-Aldrich, St Louis, MO) just before the start of the experiment. After the experiment, cells were adhered to microscopic slides by centrifugation in a Cytospin 3 (Shandon) centrifuge and followed by fixation in ice cold acetone. Boundary of cell population was marked with glass marker and cells were blocked with 2% bovine serum albumin in PBS and stained with indicated primary antibodies overnight at 4°C, followed by Alexa Fluor 488 (Molecular Probes; Life Technologies, Grand Island, NY) florescent labeled secondary antibody for 1 hour. Nuclei were stained with DAPI (4,6 diamidino-2-phenylindole) (Vector laboratories) along with mounting medium. Images were collected using Olympus Fluoview 1000 Laser Scanning confocal microscope. Z stacks of 20 to 30 slices through the cell (0.4 μm) were collected for each slide. Images were processed with Olympus Fluoview (Version 3.0) software and represent 1 slice per slide through middle of nucleus.

2A2-IgG internalization: To examine binding and internalization of 2A2-IgG, cells were treated with 2A2-IgG- Alexa fluor-488 or CD19-FITC antibody for 20 min on ice followed by

incubation at 37^{o}C for 15 minutes. Unbound antibodies were washed away using stripping buffer and fixed with 2% paraformaldehyde for 30 min. Cells incubated with 2A2-IgG on ice without stripping buffer wash was used as a negative control. Nuclei were stained with 20 μM DRAQ5^{TM} for 5 min at room temperature. Cells mounted on glass slide were monitored and analyzed by Zeiss 510 META Laser Scanning Confocal Imaging Systems and LSM Image software (Carl Zeiss Microimaging, Inc., NY) under green and blue fluorescence for Alexa fluor-488 and DRAQ5, respectively, with 600X magnification.

In-vivo experiments:

All animal experiments were carried out under protocols approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC). C57BL/6 animals (Taconic Farm, Germantown, NY) were used for immunosuppressive studies comparing FTY720 and OSU-2S. Eμ-TCL1 mice[1] with white blood cell (WBC) count >15 x 10^3/μl were used for cytoreduction studies. Animals meeting the criterion *de-novo* were grouped into Vehicle or OSU-2S and received three daily doses (5mg/kg) of treatment and were bled to assess WBC count by staining peripheral blood smear. CD19 and CD3 percentages were analyzed by flowcytometer after RBC depletion.

Eμ-ROR1-TCL1 mouse engraftment studies were done by injecting 10x10^6 splenocytes isolated from Eμ-ROR1-TCL1 double transgenic animal with known frank leukemia and splenomegaly into Eμ-ROR1 single transgenic mouse by tail vein injection for leukemia adaptation. Upon confirmation of leukemia as evidenced by circulating CD5+CD19+ cells and splenomegaly, the splenic cells were engrafted into syngenic C57BL/6 animals. Splenocytes isolated by ficoll

density centrifugation were stained for B220, CD5, hROR1 expression and viability and about $10x10^6$ cells were injected into tail vein of C57BL/6 recipient and the disease progression was monitored bi-weekly by flowcytometric analysis of peripheral blood. Animals that have developed high WBC count (>$10x10^3$/µl) and 5% B220+CD5+ leukemic cells in peripheral blood were grouped into treatment groups and dosed 10 mg/kg everyday by intra-peritoneal injection of ILP formulations. All animals were monitored for signs of disease and other early removal criteria, including greater than 20% weight loss or cancer induced cachexia, inability to feed or move around and other infections.

Eµ-ROR1 transgenic mice generation:

A 3.5kb *Not1* human ROR1hROR1 cDNA fragment from pCMV6-XL6 plasmid (kindly provided by Christoph Rader, NCI) was cloned into the *Bgl2* site of the pBH vector that was shown to direct B cell specific expression of transgenes.[2, 3] Expression of hROR1 was confirmed in 70/z3 mouse pre-B cell lines transfected with the above construct containing hROR1 cDNA. Transgenic mice were generated by pronuclear injection of about 6.5kb fragment released with *Not1/Kpn1* from the pBH transgenic construct in fertilized oocytes from C57BL/6 animal at Genetically Engineered Mouse Modeling facility at the Ohio State University Comprehensive Cancer Center (OSUCCC). The founders were identified by southern blot analysis of tail DNA for the presence of hROR1 transgene using P^{32} labeled hROR1 cDNA probe (fragment size and restriction enzyme used to generate the probe). Two independent founder animals identified were bred to C57BL/6 to establish founder lines that were genotyped by PCR with primers specific for hROR1 using genomic tail DNA.

Selectivity study:

Cell lines or human PBMC cells were co-incubated with 2A2-IgG followed by secondary PE-conjugated anti-mouse IgG antibody (Abcam, Cambridge, MA), as well as either CD19-FITC or CD3-FITC antibodies on ice for 30 min. Cells incubated with 2A2-ILP calcein at 37^{o}C for 30 min were then spun down and rinsed with cold PBS twice and stained with CD19-PE or CD3-PE on ice to identify B and T cell populations, respectively. Cells were then washed with PBS and analyzed by flow cytometry using Kaluza analysis program (Beckman-Coulter, Inc., Brea, CA).

Single cell suspension of spleens from ROR1 transgenic and non-transgenic mouse were RBC lysed using RBC lysis buffer (154.95 mM NH_4Cl, 10mM $KHCO_3$, 0.01mM EDTA) on ice for 10 minutes and PBS washed and incubated with 2A2-ILP FAM ODN at 37^{o}C for 30 minute, washed twice with PBS and stained with anti-mouse B220-PE identify B cells. Biotinlyated anti-hROR1 antibody and corresponding avidin PE secondary antibody were used as controls. Non-transgenic littermates or C57Bl/6 mice were used as controls for transgenic animals as needed.

Internalization study:

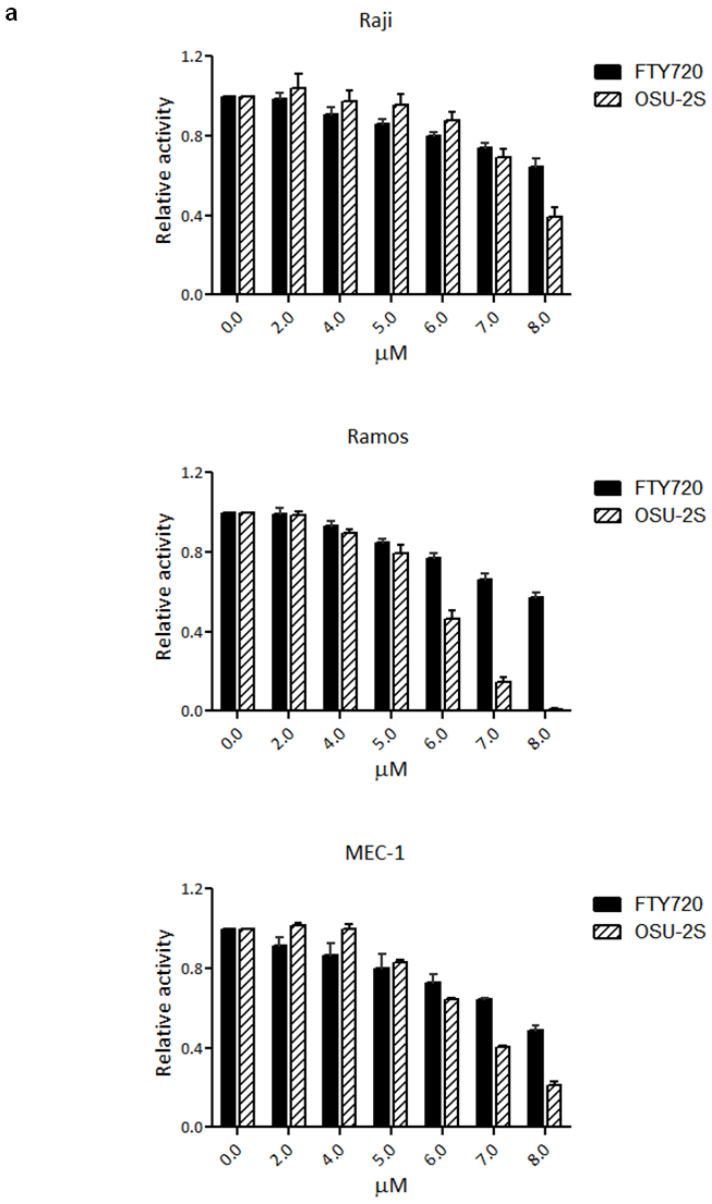
The internalization rate of antibodies was determined by measuring and comparing the mean fluorescent intensity of fluorescently-labeled antibodies as described. Two million cells were stained with approximately 1μg/mL antibodies on ice for 30 min in PBS. Cells were washed twice with ice-cold PBS and incubated at 37^{o}C for indicated time points from 15 min to 4 hr. At each time point, cells were placed on ice and surface-bound antibodies were removed by 500 μl of stripping buffer (100 mM glycine, 100 mM NaCl (pH 2.5)) at room temperature for 2 min before washing twice with ice-cold PBS. Appropriate IgG isotypes were used as negative

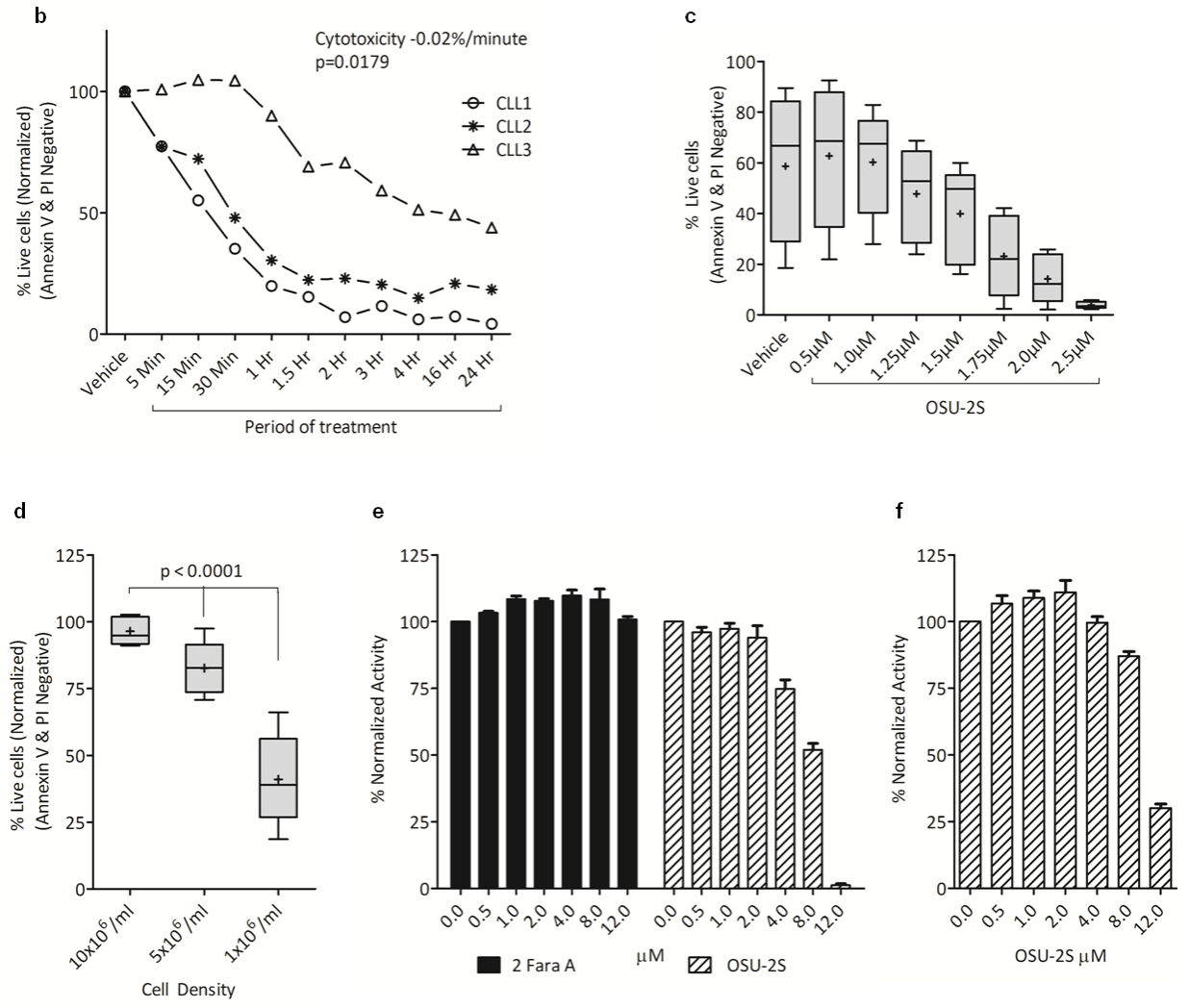
controls. Internalization is defined as time-dependent increase in the mean fluorescent intensity (MFI) detected by flow cytometry after removal of any surface bound antibodies and normalized to MFI at 30 min on ice as the 100% antibody expression on the cell surface.

Statistics:

For data with repeated measures, mixed effect models were used considering observational dependencies across subjects.[4] Matched samples were compared by paired t-tests. Analysis of variance (ANOVA) was used to compare means of multiple independent groups. The association between phospho SHP1 and change in cell viability was assessed by Spearman correlation test. The log-rank test was applied for analysis of animal survival study. Holm's method was employed to adjust multiplicity to control the family wise error rate at 0.05.[5] For the gene expression profiling experiment, data were normalized by RMA method and two-sample t-tests were used to detect differentially expressed genes. Smoothing method was applied to improve variance estimates in the tests[6]. The expected false positive rate was controlled at 0.0005 (five false positive out of 10000 tests).[7]

Supplementary Figure S1

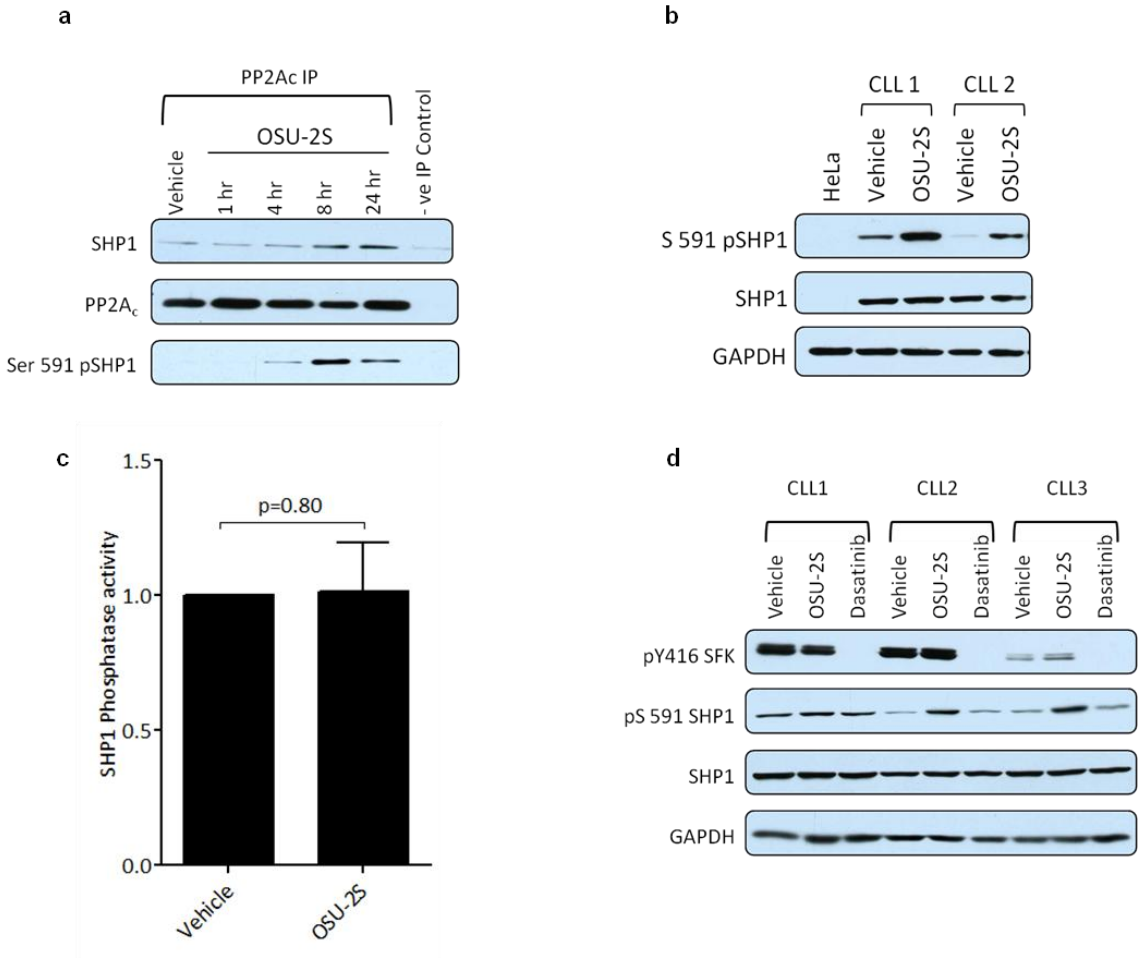


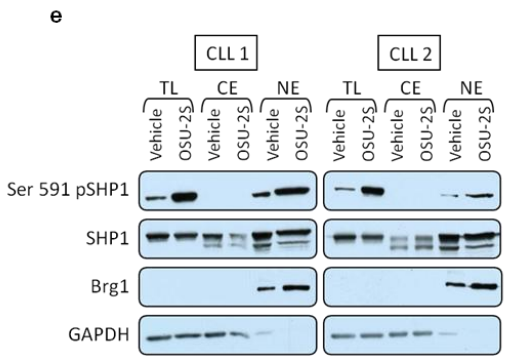


Supplementary Figure S1: OSU-2S mediates cytotoxicity in cell lines and in CLL primary cells. (a) Comparison of cytotoxic profiles of FTY720 and OUS-2S in various B cell lines. MTS cell proliferation assay was done on cell lines (Raji, Ramos, MEC-1) ($1x10^6$/ml) treated with indicated concentrations of FTY720/OSU-2S for 24hrs. Values were normalized to vehicle treated conditions. Figures represent mean with SD of three independent experiments. **(b-d)** Cytotoxic effect of OSU-2S in CLL by time kinetics; dose kinetics; cell density. Freshly isolated CD19+ CLL primary cells were incubated with indicated concentrations of OSU-2S and the

viability was assessed 24 hours later by annexin-V FITC and PI staining. **(b)** Time kinetics: CLL cells ($10x10^6$/ml) (N=3) were treated with 8µM OUS-2S for indicated duration, washed and replated in RPMI 1640 medium and viability was assessed 24 hours later after initial treatment. **(c)** Dose kinetics: CLL cells ($1x10^6$/ml) (N=6) were treated with varying doses of OSU-2S and viability was assessed 24 hours later. **(d)** Cell density dependency: CLL cells of different seed density ($10x10^6$/ml; $5x10^6$/ml and $1x10^6$/ml) (N=5) were treated with 2µM OSU-2S and viability was assessed 24 hours later. **(e-f)** OSU-2S abrogated the growth of fludarabine (2FaraA) resistant MEC-2 cells **(e)** and rituximab resistant Raji cell line clones 2R **(f)**. Cell lines were treated with different concentrations of OSU-2S for 48hrs and proliferation activity was measured by MTS assay. Values were normalized to vehicle treated conditions. Figures represent mean with SD of three independent experiments.

Supplementary Figure S2

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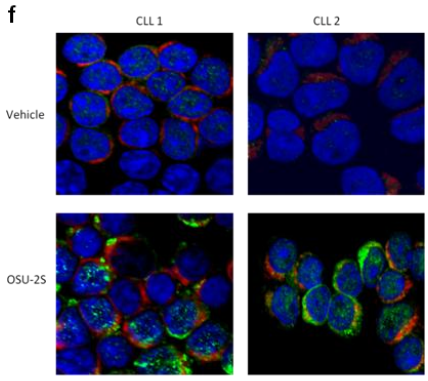
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Vehicle
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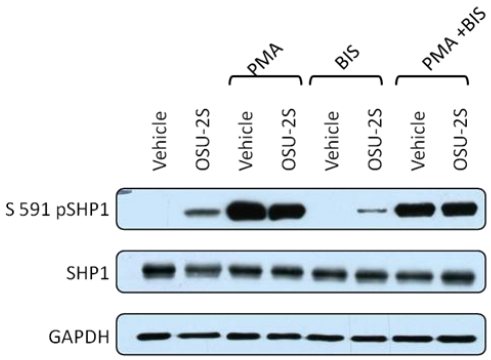
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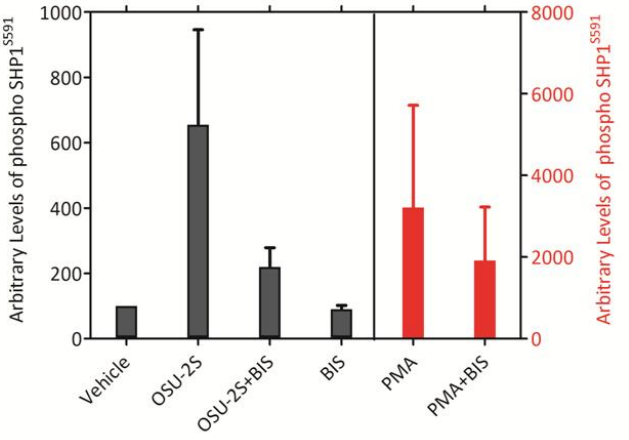
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Arbitrary Levels of phospho SHP1S591
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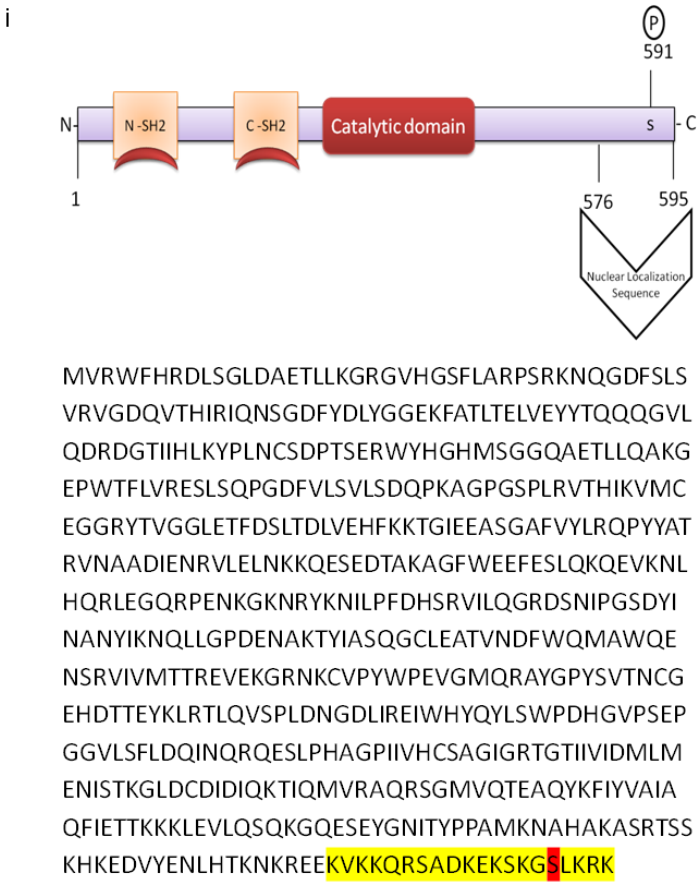
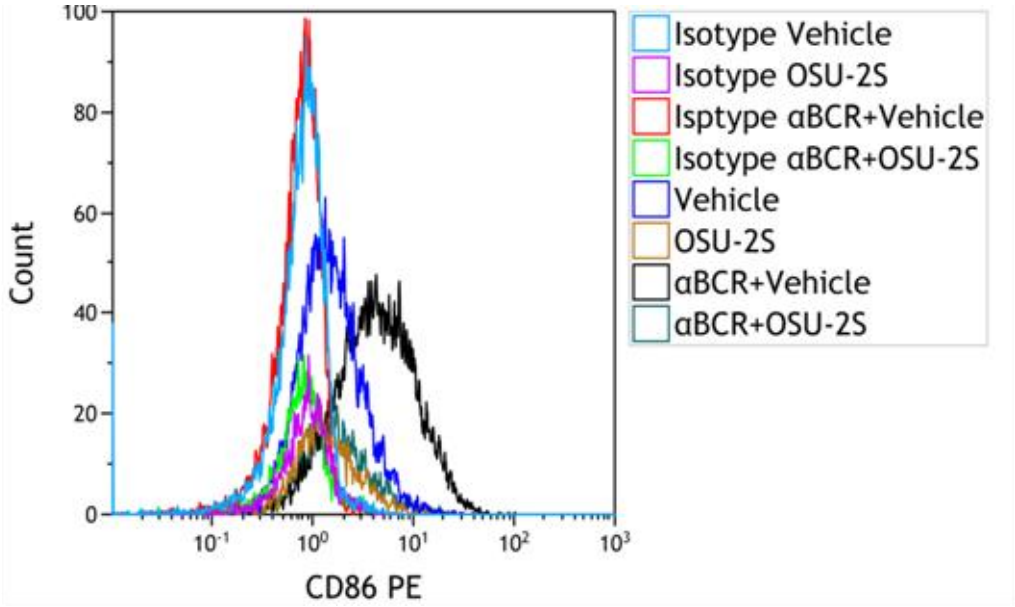


Supplementary Figure S2: OSU-2S induces SHP1^{S591} phosphorylation in CLL cells.

(a) Association of PP2A and SHP1. PP2A$_c$ was immunoprecipitated from CLL cells treated with OSU-2S(8µM) at different time points and probed for PP2A$_c$, SHP1 and phospho SHP1^{S591}. The blot is representative of 3 independent experiments. Mouse IgG was used as negative IP control. **(b)** OSU-2S induces phosphorylation of SHP1^{S591} at the putative PKC substrate motif (K/RXS*XK/R) on SHP1. CLL cell lysates were made after 5 hours of treatment with OSU-2S and immunoblotted for SHP1, phospho SHP1^{S591} and GAPDH. **(c)** SHP1^{S591} phosphorylation does not affect enzyme activity of SHP1 in CLL. Vehicle or OSU-2S treated CLL cells were lysed at 5 hours after treatment and SHP1 was immunoprecipitated from cell lysates and used for *in-vitro* SHP1 phosphatase enzyme assay by incubating with phospho tyrosine substrate and the released free phosphate was measured using malachite green solutions and reading absorbance at

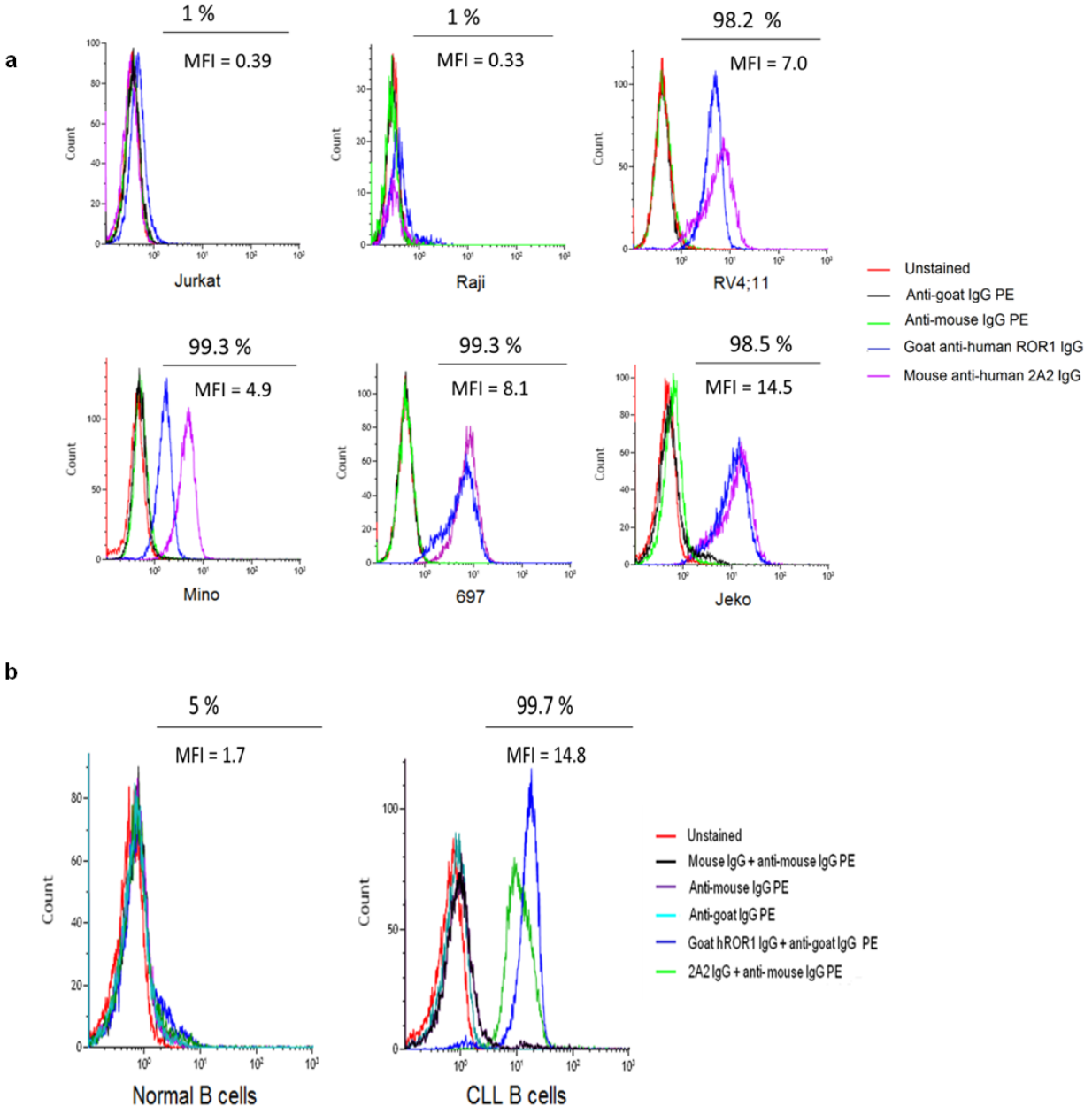
620nm. Values were normalized to protein levels and vehicle treated control. Bars represent SD. (N=5). **(d)** OSU-2S does not affect SFK ^{Y416} phosphorylation. OSU-2S treated CLL cell lysates were immunoblotted for phospho SFK $^{Y416.}$ **(e)** Nuclear localization of phospho SHP1^{S591}. Total lysates (TL), cytosolic extract (CE) and nuclear extract (NE) fractions were isolated from Vehicle or OSU-2S treated CLL cells at 5 hour time point and immunoblotted for SHP1, phospho SHP1^{S591}, GAPDH and BRG-1. Shown is the representative blot of two CLL patients from N=8. **(f)** Confocal fluorescence microscopic image of phospho SHP1^{S591}. Vehicle or OSU-2S treated CLL cells were fixed onto slides at 5 hour time point and were stained for cell membrane (red), phospho SHP1^{S591} (green) and nuclei (blue) and imaged using Olympus Fluoview 1000 laser scanning confocal microscope with Z stacks of 0.4µm per slice and images were chosen from the middle of nuclei. The image is representative of N=5 CLL patient samples. **(g)** PKC inhibitor BIS reduces phospho SHP1^{S591}. Immunoblotting for phospho SHP1^{S591} in CLL cells treated with Vehicle or OSU-2S (5 hours) in the presence or absence of PKC inhibitor 2µM BIS. PMA is used as control for BIS. Figures are representative of N=8 CLL patient samples. **(h)** Quantification of immunoblot signal from previous experiments using Chemi-Doc system with Quantity one software. Y-axis represents mean with SE of arbitrary numbers normalized to vehicle control (N=5). **(i)** Schematic diagram of SHP1 protein and its amino acid sequence showing the C-terminal serine 591 residue (highlighted red) and the putative nuclear localization sequence (highlighted yellow).

Supplementary Figure S3



Supplementary Figure S3: OSU-2S inhibits B-cell receptor (BCR) mediated activation and expression of activation marker CD86 in CLL cells. CLL cells were stimulated with 6.5µg/ml goat F(ab')2 against human IgA+IgG+IgM (H+L) 30 minutes before addition of vehicle or OSU-2S(8µM). Viability and CD86 expression was assessed after 24 hrs by flowcytometry.

Supplementary Figure S4



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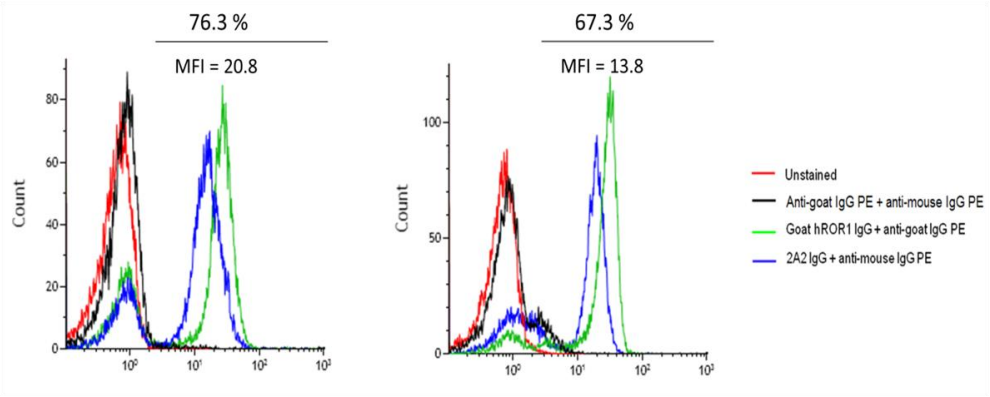
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MFI = 20.8
67.3 %
MFI = 13.8
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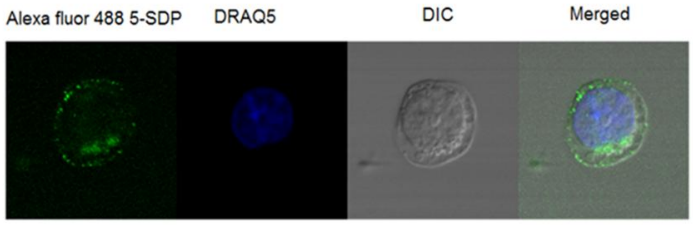
Goat hROR1 IgG + anti-goat IgG PE
2A2 IgG + anti-mouse IgG PE

d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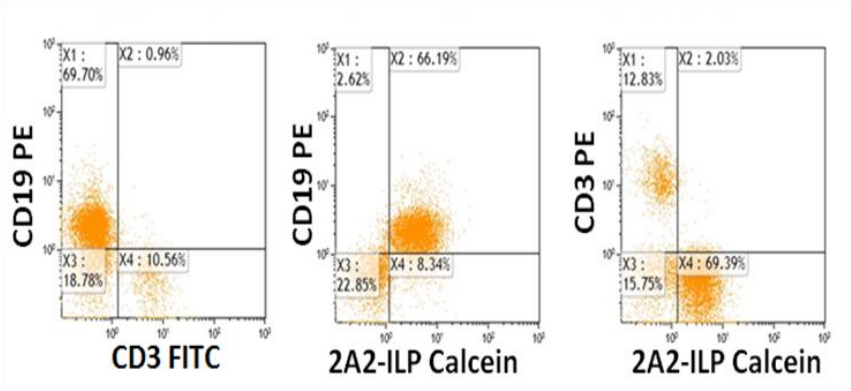
Merged

e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X2 : 0.96%
X3 : 18.78%
X4 : 10.56%
CD19 PE
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X2 : 66.19%
X3 : 22.85%
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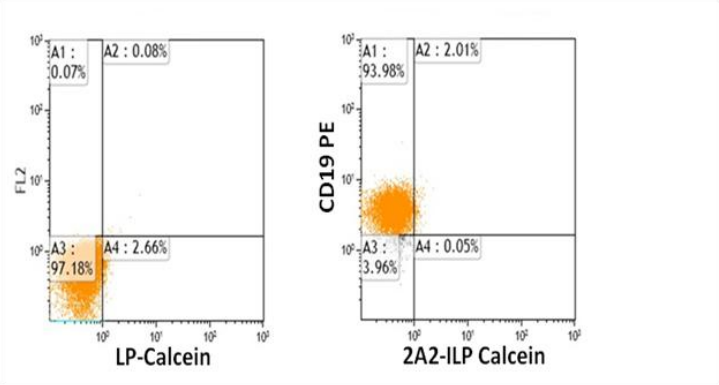
X4 : 69.39%

f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A3 : 97.18%
A4 : 2.66%
CD19 PE
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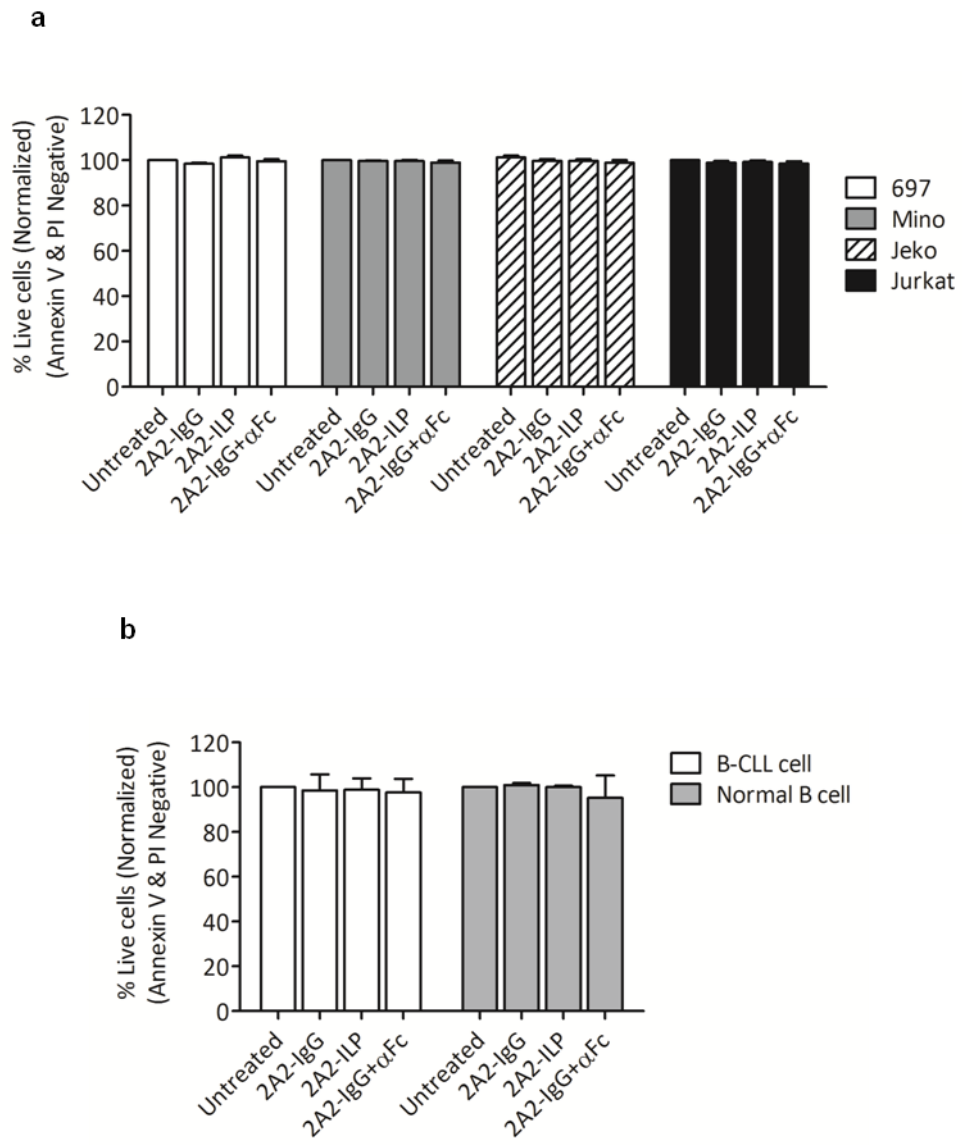
A2 : 2.01%
A3 : 3.96%
A4 : 0.05%

Supplementary Figure S4: ROR1 surface expression in CLL and binding of 2A2-IgG to CLL. (a-c) 2A2-IgG targets hROR1+ B cells. Cell surface expression of hROR1 in cell lines, B cells and PBMC from CLL patients was analyzed using 2A2-IgG and goat anti-hROR1 polyclonal antibodies. 1×10^6 cells were co-incubated with 2A2-IgG or control antibody followed by PE-conjugated secondary antibody on ice for 30 min. Cells were then washed with PBS and analyzed by flow cytometry. **(a)** Surface expression of hROR1 in different cell lines Jurkat, Raji, RV4;11, Mino, 697 and JeKo. **(b)** Surface expression of hROR1 in normal B cells from healthy donors and CLL cells. **(c)** Surface expression of hROR1 in PBMC from two CLL patients. **(d)** Confocal microscopic image of cells treated with Alexa fluor-488 conjugated 2A2-IgG for 20 min on ice followed by incubation at 37°C for 15 min. Unbound antibodies were washed away by stripping buffer and fixed with 2% paraformaldehyde for 30 min. Cells incubated with 2A2-IgG on ice without stripping buffer wash was used as a negative control. Nuclei were stained with 20 μM DRAQ5^{TM} for 5 min at room temperature. Cells mounted on glass slide were monitored and analyzed by Zeiss 510 META Laser Scanning Confocal Imaging Systems and LSM Image software (Carl Zeiss Microimaging, Inc., NY) under green and blue fluorescence for Alexa fluor-488 and DRAQ5, respectively, with 600X magnification. **(e)** PBMC from CLL patients (N=8) and **(f)** normal B cells from healthy donors (N=8) showing the binding and uptake of 2A2-ILP calcein by CLL cells but not normal B cells.

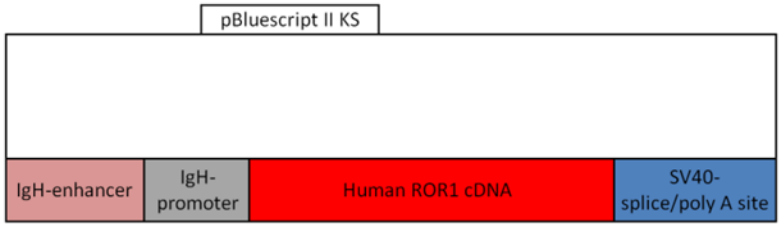
Supplementary Figure S5



Supplementary Figure S5: 2A2-IgG does not induce direct cytotoxicity in ROR1+ B cells. 1×10^6 cells were treated with the indicated agents at 1.5 µg/mL of 2A2-IgG for 24 hr and viability was analyzed by flow cytometry. Results were normalized to the untreated group; **(a)** Cell lines 697, Mino and JeKo positive for ROR1 were used. Jurkat (ROR1-ve) used as negative control. (N=3, mean ± SD, P>0.05) **(b)** Primary CLL cells and normal B cells (N=6, mean ± SD, P>0.05).

Supplementary Figure S6

a



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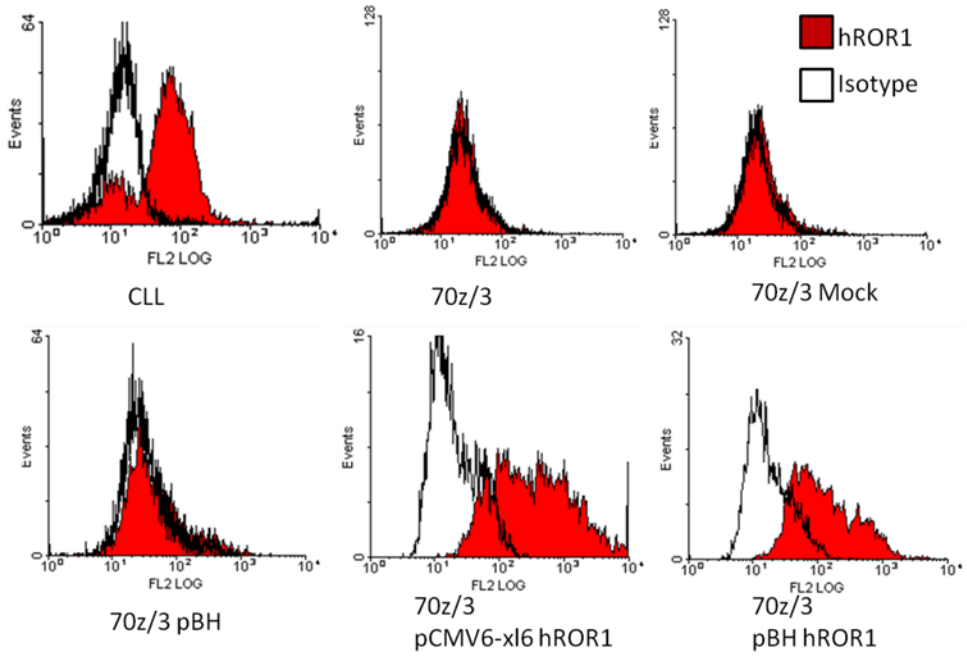
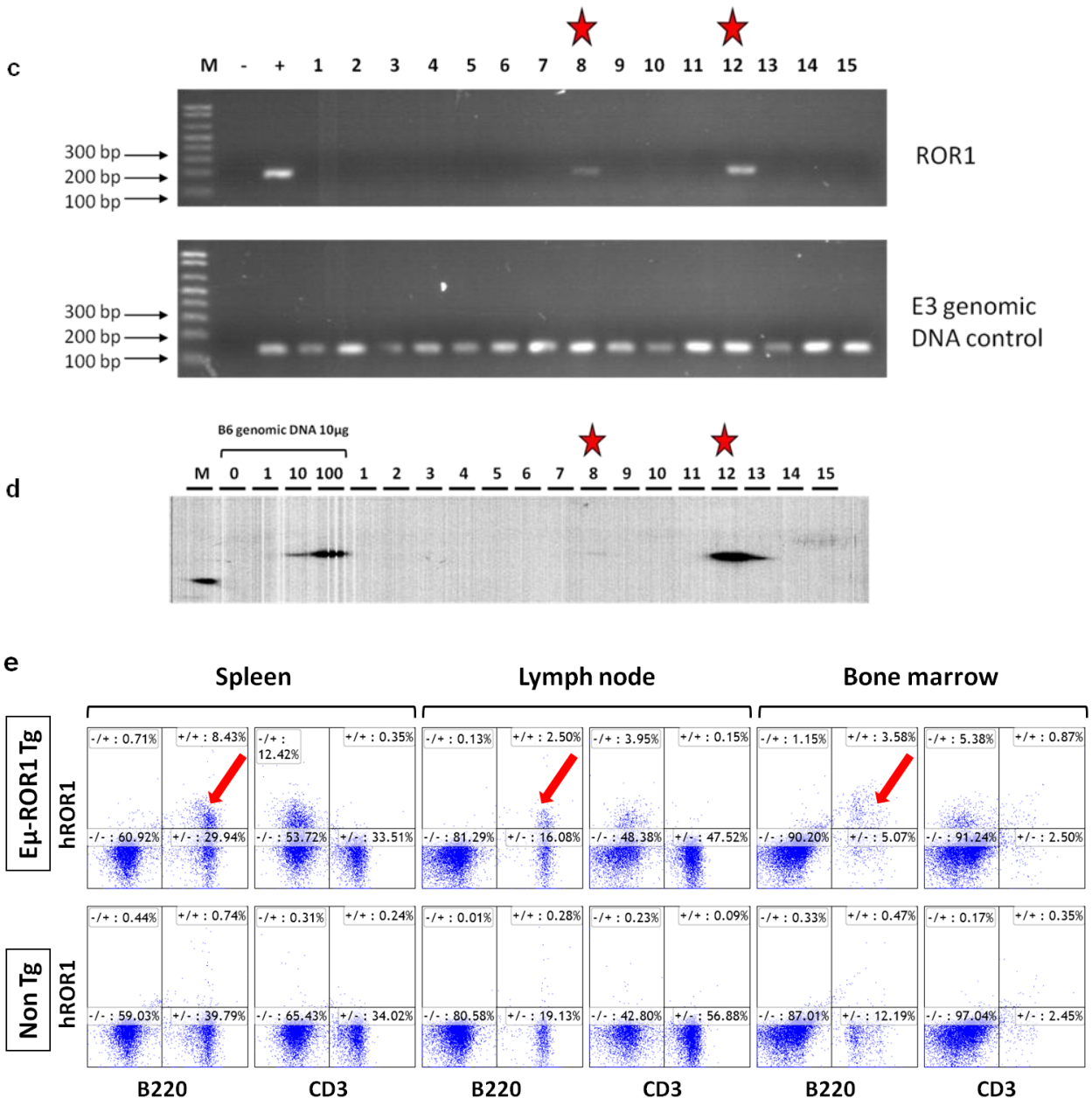
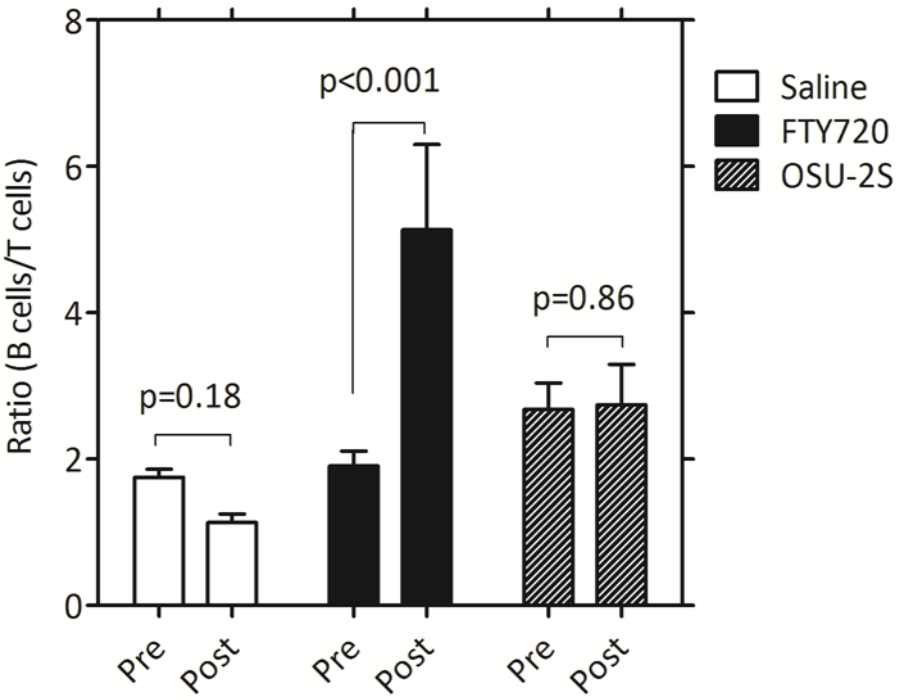




Supplementary Figure S6: Eμ-ROR1 transgenic (Tg) mice generation. (a) pBH hROR1 plasmid construction map. hROR1 cDNA cloned into pBH between IgH promoter/enhancer and SV40 poly A site transcriptional elements. **(b)** pBH hROR1 plasmid expression in 70z/3 mouse pre-B cell line. 70z/3 cells were transfected with mock, vector or hROR1 containing pBH construct and stained for cell surface hROR1 with goat anti-hROR1 polyclonal antibody and

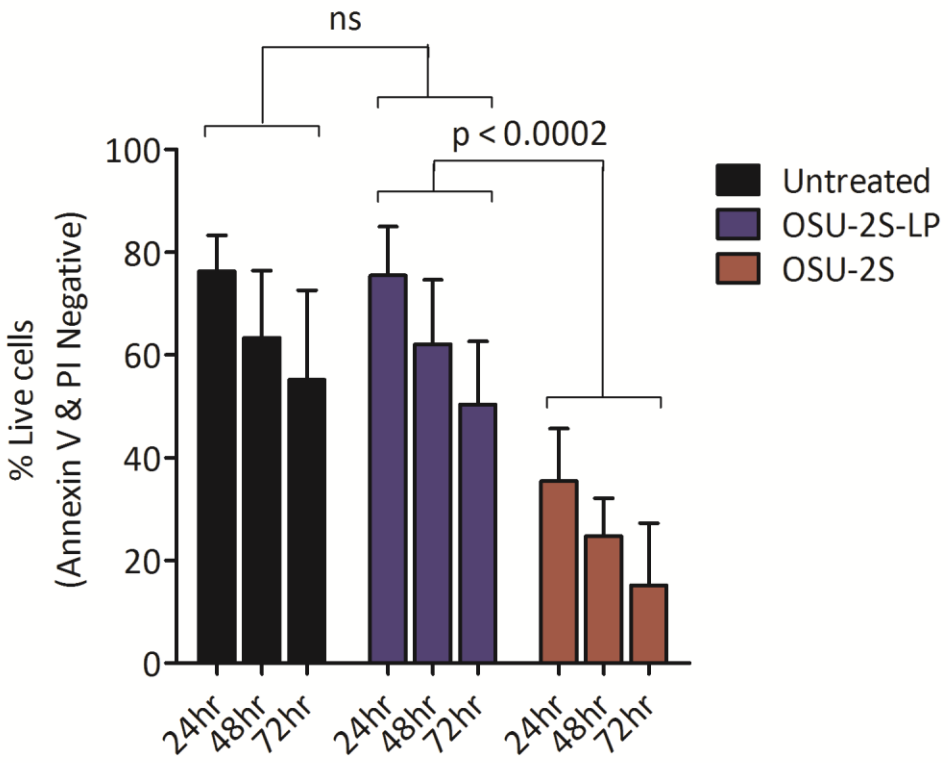
analyzed by flow cytometry 48hrs post transfection. **(c)** ROR1 Tg mouse founders PCR genotyped using primers specific for hROR1 cDNA. **(d)** ROR1 Tg mouse founders confirmation by Southern blotting using P^{32} labeled *Dra2* probe containing partial hROR1 cDNA sequence. **(e)** hROR1 expression in Eμ-ROR1 Tg mice. Spleen, lymph node and bone marrow from hROR1 Tg mice and age, sex matched non Tg mice were stained for hROR1 using goat anti-hROR1 polyclonal antibody and B220 or CD3 and analyzed by flow cytometry. (Representative of N=3).

Supplementary Figure S7



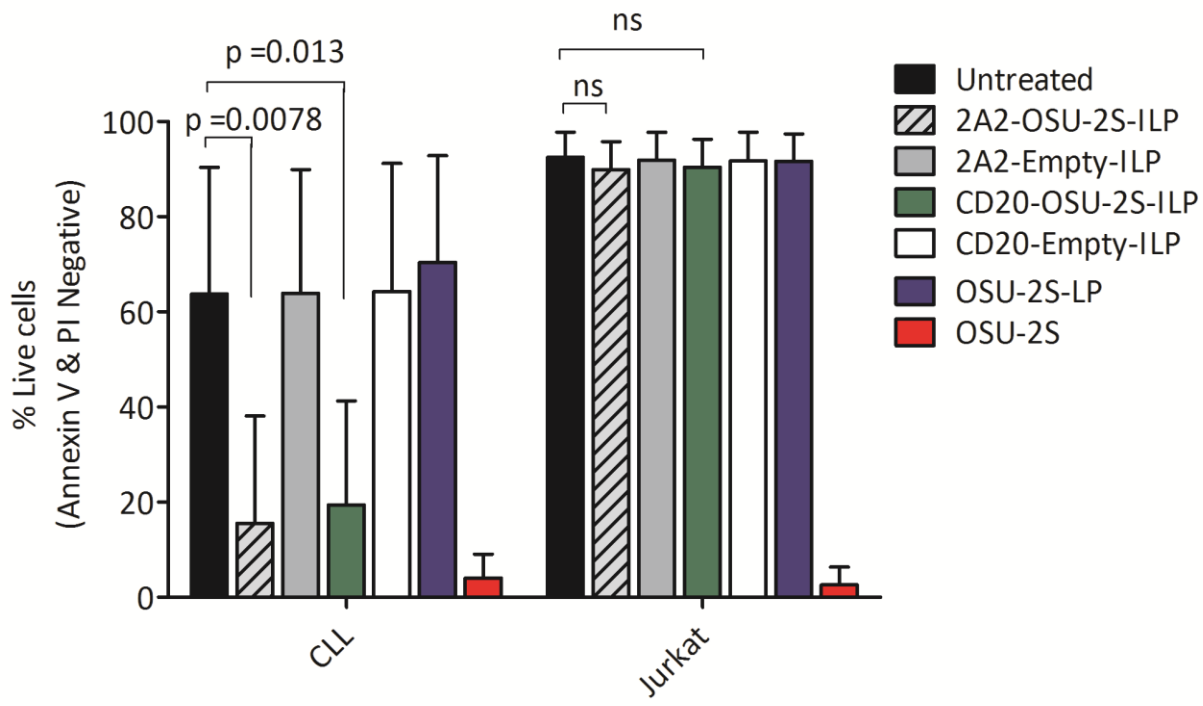
Supplementary Figure S7: OSU-2S does not affect T cell trafficking. Administration of FTY720 (5mg/kg) but not OSU-2S(5mg/kg) decreased the peripheral blood T cell count in C57BL/6 mice. Lymphocyte counts were expressed B-to-T-cell ratios to avoid misinterpretation of cytotoxic effects and cell trafficking effect. The B-to-T-cell ratio remained unchanged before and after treatment with saline or OSU-2S but increased in FTY720 treated animals due to a decrease in peripheral T cell numbers. Mice peripheral blood samples were collected and stained for B220 and CD3 surface markers before and 6 hours after administration of the above reagents.

Supplementary Figure S8



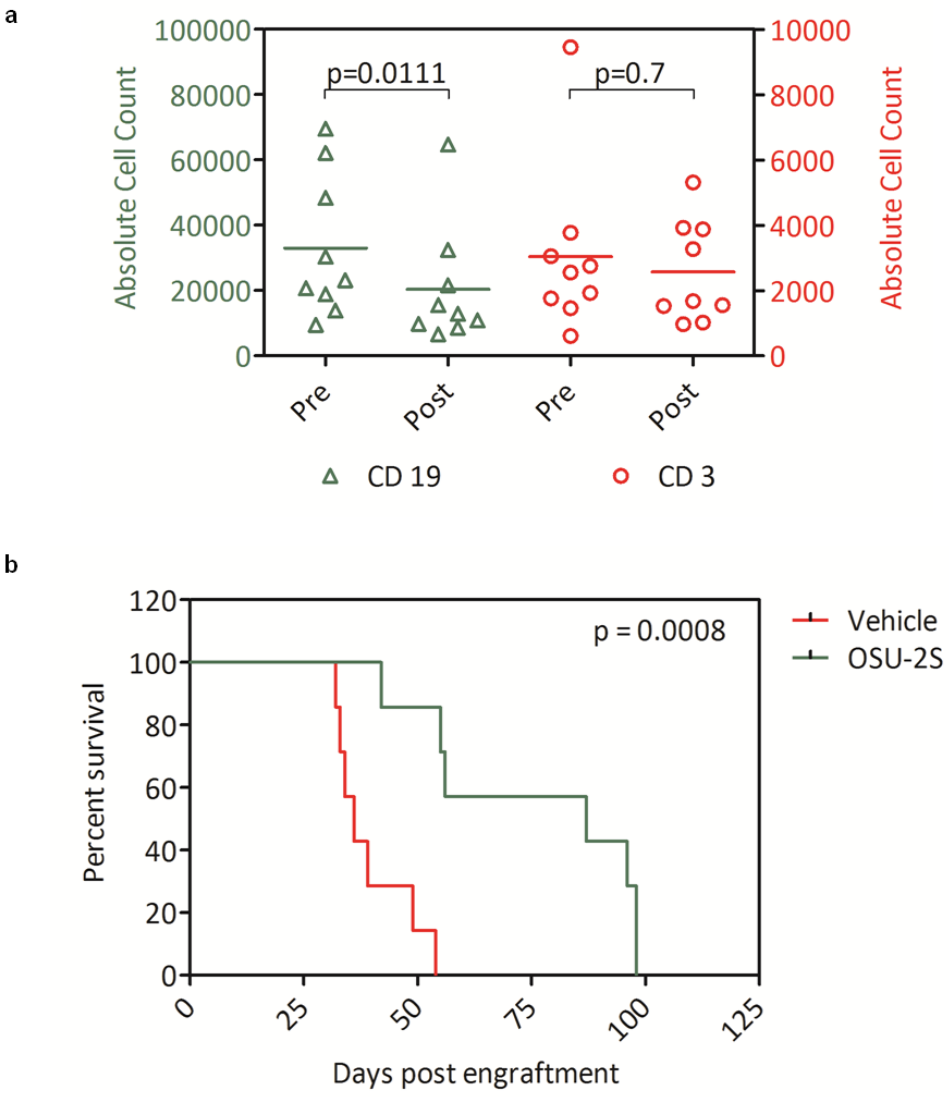
Supplementary Figure S8: Long term effect of OSU-2S-LP on normal B cells. Normal B cells (1×10^6 per ml, N=4 donors) isolated from healthy donors were incubated with OSU-2S-LP formulation at 5μM of OSU-2S or free OSU-2S (5μM) for indicated duration before viability was analyzed by flow cytometry. Free OSU-2S is cytotoxic to normal B cells but no cytotoxicity by OSU-2S-LP formulation.

Supplementary Figure S9



Supplementary Figure S9: 2A2-OSU-2S-ILP promoted comparable levels of cytotoxicity as CD20-OSU-2S-ILP in CLL. CLL cells (1×10^6 per ml, N=4 patients) or Jurkat (1×10^6 per ml, N=4) were incubated with different ILP formulation at 5μM of OSU-2S and 0.1μg/ml mAbs or free OSU-2S (5μM) for 24 hr before viability was analyzed by flow cytometry. ROR1 targeting (2A2-OSU-2S-ILP) and CD20 targeting (CD20-OSU-2S-ILP) OSU-2S formulations have comparable levels of cytotoxicity in CLL cells (no difference between 2A2-OSU-2S-ILP and CD20-OSU-2S-ILP), but no cytotoxicity in ROR1-ve CD20-ve Jurkat cells.

Supplementary Figure S10



Supplementary Figure S10: OSU-2S is active in leukemic mouse models of CLL. (a) Eμ-TCL1 mice (N=9) with WBC count >15X10^3/μl were treated with low doses of OSU-2S (5mg/kg, intra-peritoneal injection) daily for three days. WBC count, B-cell (CD19) and T-cell (CD3) percentiles were determined before initial dosing and 24 hours after last dosing. Absolute cell numbers per micro liter of blood are shown. **(b)** Survival curve for Eμ-ROR1-TCL1 double transgenic splenocytes engraftment model of CLL. 10x10^6 Eμ-ROR1-TCL1 double transgenic

splenocytes were engrafted into syngenic C57BL/6 animals and disease progression was monitored bi-weekly by flowcytometric analysis of peripheral blood for WBC count. Animals were grouped two weeks after engraftment and dosed 5 days every week for 4 weeks by intra-peritoneal injection of vehicle (HPβCD) or OSU-2S as single agent (10mg/kg) (N=7/group).

Supplementary Table 1:

Characterization of OSU-2S-LP and 2A2-OSU-2S-ILP.

Formulation	Particle size (nm)	Polydispersity index (PI)	Entrapment Efficiency (EE) (%)	Zeta potential (mV)
Empty liposome	159.1 ± 5.40	0.186 ± 0.038	N/A	- 4.10 ± 0.34
OSU-2S-LP	157.2 ± 3.98	0.168 ± 0.056	90.09 ± 2.69	5.41 ± 1.12
2A2-OSU-2S-ILP	163.3 ± 4.31	0.184 ± 0.043	88.25 ± 4.81	0.32 ± 1.75

Supplementary Table 2:

ROR1 expression by microarray (gene expression) and flowcytometry (cell surface) on CLL cells after OSU-2S treatment.

Probe set	Gene expression Fold change to vehicle	P value
ROR1 Probe1	1.067288348	0.262766
ROR1 Probe2	0.933725248	0.275637
ROR1 Probe3	0.92999831	0.265262

Cell surface ROR1	**6hr** (ns)	**18hr** (ns)
	ΔMFI	**ΔMFI**
CLL1	-0.42	0.43
CLL2	-0.68	0.69
CLL3	-0.05	1.86
CLL4	-0.13	-0.89

ns - not significant

Supplementary Table 3:

CD19 and CD20 cell surface expression on CLL cells after OSU-2S treatment.

Cell surface CD19	**6hr** (ns)	**18hr** (ns)
	ΔMFI	**ΔMFI**
CLL1	-0.79	-2.41
CLL2	-0.99	-4.12
CLL3	-0.24	-1.72
CLL4	-4.29	-6.36

ns - not significant

Cell surface CD20	**6hr** (ns)	**18hr** (ns)
	ΔMFI	**ΔMFI**
CLL1	-6.03	-6.57
CLL2	-13.93	-16.55
CLL3	-6.83	-5.45
CLL4	-30.75	-54

ns - not significant

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