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

Rajeswaran Mani^{1,2,3,†}, Yicheng Mao^{2,4,5,†}, Frank W. Frizzera^{1,2}, Chi-Ling Chiang^{1,2}, Jiang Wang², Yuan Zhao⁴, Yun Wu⁵, Bo Yu^{2,5}, Ribai Yan⁶, Xiaokui Mo⁷, Lianbo Yu⁷, Joseph Flynn¹, Jeffrey Jones¹, Leslie Andritsos¹, Sivasubramanian Baskar⁸, Christoph Rader⁹, Mitch A Phelps^{2,4}, Ching-Shih Chen^{2,3,6}, Robert J. Lee^{2,4,5}, John C. Byrd^{1,2,3,6}, L. James Lee^{5,10}, and Natarajan Muthusamy^{1,2,3,*}

¹Division of Hematology, Department of Internal Medicine, College of Medicine, The Ohio State University, Columbus, OH, USA

²Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA

³Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA

⁴Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, OH, USA

⁵Center for Affordable Nanoengineering of Polymeric Biomedical Devices, The Ohio State University, Columbus, OH, USA

⁶Division of Medicinal Chemistry, College of Pharmacy, The Ohio State University, Columbus, OH, USA

⁷Center for Biostatistics, The Ohio State University, Columbus, OH, USA

⁸Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

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***Corresponding Author:** Natarajan Muthusamy DVM., Ph.D, Associate Professor of Medicine, Division of Hematology, Dept. of Internal Medicine, Mol. Virology Immunology and Medical Genetics and Vet. Biosciences, The Ohio State University Comprehensive Cancer Center, **Address:** 455E, OSUCCC, 410, West 12th Avenue, Columbus, OH 43210 USA, Ph: (614) 292-8135, Fax: (614) 292-3312, raj.muthusamy@osumc.edu.

[†]These two authors contributed equally to this work.

AUTHOR CONTRIBUTIONS: R.M. planned, designed and performed majority of the *in-vitro* and *in-vivo* research, analyzed data, wrote the initial and subsequent drafts of the paper and approved the final version of the paper. Y.M. synthesized and optimized targeted liposomes, designed and performed research, analyzed data, wrote the initial drafts of the paper. F.F. generated and characterized the ROR1 and ROR1xTCL1 double transgenic mice, assisted with the experiments. C.C., J.W., Y.Z., Y.W., and B.Y. synthesized and characterized targeted liposomes, helped with experiments. R.Y. synthesized OSU-2S. X.M. and L.Y. participated in designing sample sizes for each experiment, performed the statistical analysis. J.C.B., J.F., J.J., and L.A. contributed to the CLL patient care, patient sample acquisition and characterization, translation insight to the experimental designs. S.B., C.R., M.A.P., R.J.L. and C.S.C. and L.J.L. provided necessary reagents, supported components of the research. N.M. oversaw the study, sought funding, participated in experimental design and generation of mouse models, data interpretation.

CONFLICT OF INTEREST: A provisional patent has been filed for the use of OSU-2S to treat hematologic malignancies. The authors declare no additional competing financial interests.

Supplementary information is available at *Leukemia's* website.

⁹Department of Cancer Biology and Department of Molecular Therapeutics, The Scripps Research Institute, Scripps Florida, Jupiter, FL, USA

¹⁰Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH, USA

Abstract

Selective cytotoxicity to cancer cells without compromising their normal counterparts pose a huge challenge for traditional drug design. Here we developed a tumor antigen targeted delivery of immunonanoparticle carrying a novel non-immunosuppressive FTY720 derivative OSU-2S with potent cytotoxicity against leukemic B cells. OSU-2S induces activation of protein phosphatase 2A, phosphorylation and nuclear translocation of SHP1^{S591} and deregulation of multiple cellular processes in chronic lymphocytic leukemia (CLL) resulting in potent cytotoxicity. To preclude OSU-2S mediated effects on these ubiquitous phosphatases in unintended cells and avoid potential adverse effects we developed a OSU-2S targeted delivery immunonanoparticles (2A2-OSU-2S-ILP), that mediated selective cytotoxicity of CLL but not normal B cells through targeting receptor tyrosine kinase ROR1 expressed in leukemic but not normal B cells. Developing a novel spontaneous CLL mouse model expressing human ROR1 (hROR1) in all leukemic B cells, we demonstrate the therapeutic benefit of enhanced survival with 2A2-OSU-2S-ILP *in-vivo*. The newly developed non-immunosuppressive OSU-2S, its delivery using human CLL directed immunonanoparticles and the novel transgenic mouse model of CLL that expresses hROR1 exclusively in leukemic B cell surface are highly innovative and can be applied to CLL and other ROR1+ malignancies including mantle cell lymphoma and acute lymphoblastic leukemia.

Keywords

CLL; OSU-2S; phosphatases; ROR1; immunonanoparticle; ROR1 transgenic mice

INTRODUCTION

Chronic lymphocytic leukemia (CLL) characterized by accumulation of apoptotic resistance CD19+CD5+ B lymphocytes in peripheral blood and lymphoid organs is the most prevalent adulthood leukemia in the western hemisphere.^{1, 2} Current therapies for CLL are not curative rendering drug adverse effects and immunosuppression.³ Several hematological malignancies including CLL display aberrantly phosphorylated proteins that have led to development of inhibitors of kinases such as SYK, BTK, PIM, mTOR and PI3K for CLL therapy.⁴ While this approach is very promising, limitations associated with simultaneously targeting multiple survival kinases involved in diverse survival signaling pathways, kinase independent survival signals and selective inhibition in leukemic but not normal B cells that are critical for the normal physiology warrant alternate approaches. The discovery of predominantly inactive phosphatases in a variety of cancers⁵⁻⁹ and the potential for phosphatase targeted therapy as an alternative to kinase inhibitors especially in situations where the efficacy of the kinase inhibitors are compromised due to resistance mechanisms attributed to mutations and single nucleotide polymorphisms of the drug targets prompted us to evaluate potential activators of phosphatases in CLL and other B cell malignancies. We

have recently reported the cytotoxic activity of FTY720, a sphingosine analogue against several B cell malignancies including CLL through modulation of protein phosphatase 2A (PP2A).^{10–12} Despite its promising *in-vitro* and *in-vivo* activity against a variety of solid tumors and heme-malignancies,^{10–15} our attempts to develop FTY720 for oncology indications was hindered by its immunosuppressive property involving T cell sequestration to lymph nodes through its action on sphingosine-1-phosphate receptor (S1PR). This prompted us to develop OSU-2S, {(S)-2-amino-2-(4-((6-methylheptyl)-oxy)phenethyl)pentan-1-ol} a synthetic derivative of FTY720 by structure activity relationship with more potent anti tumor activity but lacking the sphingosine-1-phosphate receptor mediated immunosuppressive effects.¹⁶ The promising preclinical activity of OSU-2S in prognostically poor CLL patient cells prompted us to evaluate this molecule for further studies. Prolonged exposure of unintended normal cells to chemotherapeutic drugs contribute to major adverse effects. Novel therapy options that specifically target leukemic B cells sparing normal B cells are lacking and will be highly promising for CLL patients. To address this, we entrapped OSU-2S in lipid nanoparticles targeting the tumor antigen ROR1. ROR1 is a receptor tyrosine kinase expressed in over 95% of CLL but not normal B cells. Its unaltered surface expression related to the severity of CLL disease,^{17, 18} and its relatively low cell surface density and internalization property makes it an attractive target for armed rather than naked monoclonal antibodies(mAbs). Liposomal immunonanoparticles(ILPs) have provided a modality for high interactive affinities with target antigens on cell surface and are used to selectively enhance drug payload to target cells, favorably alter the drug kinetics, overcome drug off-target effects and potentially the drug efflux pumps.¹⁹ To evaluate *in-vivo* the proof-of-principle we have generated a custom mouse model expressing hROR1 surface protein on all leukemic B cells which further closely resembled CLL. Utilizing this *in-vivo* model we show the therapeutic benefit of delivering OSU-2S loaded nanoparticles directed against hROR1 expressing leukemic cells for CLL therapy.

MATERIALS AND METHODS

Cells

Peripheral blood was obtained from CLL patients after informed consent under protocol approved by the institution's internal review board. All primary CLL cells used are immunophenotypically defined as outlined by the modified 1996 National Cancer Institute criteria.²⁰ CLL cells were isolated from fresh patient blood using "Rosette-Sep" kit (Stem Cell Technologies; Vancouver, BC, Canada) and ficoll density gradient centrifugation (Ficoll-Paque Plus; Amershan Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Normal B cells were purified from leukopaks from American Red Cross, Central Ohio (Columbus, OH). Isolated mononuclear cells were cultured in RPMI 1640 media (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO); 2 mM L-glutamine and penicillin (100 U/mL); streptomycin (100 µg/mL) (Gibco) at 37°C with 5% CO₂. Human Burkitt lymphoma cell lines Raji and Ramos were obtained from American Type Culture Collection (ATCC, Manassas, VA) and MEC-1 and MEC-2 cells were obtained from DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Mouse splenocytes were cultured as previously described²¹.

Cell viability and apoptosis

Cell death was assessed by FACS following annexin-V-FITC and propidium iodide (PI) (BD Bioscience, San Jose, CA) staining. Briefly one million cells in 200 μ l of annexin binding buffer (BD Bioscience) were stained with annexin-V-FITC and PI for 15 minutes in dark and read in Beckman-Coulter FC-500 cytometer. Proliferation of the cell lines was measured by CellTiter96[®] MTS {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} proliferation assay (Promega, Madison, WI) according to manufacturer's instruction.

Immunoblotting

Immunoblotting was done using standard methods as described previously.¹⁰ Details in supplemental information.

Phosphatase assays

Non-radioactive PP2A assay was done as previously described¹⁰ and detailed in supplemental information.

Chemicals and reagents

OSU-2S was synthesized at The Ohio State University (OSU) Medicinal Chemistry Shared Resource as described previously.¹⁶ Nuclear magnetic resonance and mass spectrometry were used to confirm the purity and identity of the compound. FTY720 was purchased from ChemieTek (Indianapolis, IN). DMSO (Gibco) was used to dissolve FTY720/OSU-2S for *in-vitro* studies. 25% (2-Hydroxypropyl)- β -cyclodextrin (HP β CD) (Sigma-Aldrich) was used to dissolve OSU-2S for *in-vivo* studies. Phorbol-12-myristate-13-acetate (PMA) (Calbiochem, Millipore, Billerica, MA); Bisindolylmaleimide (Cell Signaling Technology, Danvers, MA); Egg phosphatidylcholine (Egg PC), methoxy-polyethylene glycol (MW~2,000Da)-distearoyl phosphatidyl- ethanolamine (PEG-DSPE) were purchased from Lipoid (Newark, NJ). 2-Iminothiolane (Traut's reagent), cholesterol (Chol) and other chemicals were purchased from Sigma (St. Louis, MO). DSPE-PEG-maleimide (DSPE-PEG-mal) was obtained from Avanti Polar Lipids (Alabaster, AL). PKC activity was measured using PepTag assay kit, Promega (Madison, WI).

Antibodies against phospho S591 SHP1 (ECM Biosciences); GAPDH, SHP1, PP2Ac (Millipore); Brg1, (Santa Cruz Biotechnology, Dallas, TX); TCL1A (MBL International). Goat F(ab')₂ against human IgA+IgG+IgM (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) were commercially obtained. Anti-human CD19-FITC/PE, CD20-PE, CD3-FITC/PE, CD37-FITC, CD86-FITC and mouse B220-FITC/PE/APC-Cy7, CD19-PE, CD3-FITC/PE, CD3-PE-Cy7, CD5-FITC/PE, CD45-APC, Streptavidin-PE (BD Bioscience); hROR1 Biotinylated/ Alexa Fluor 488 (R&D Systems) were used for flowcytometric analyses. 2A2-IgG against hROR1 was from Dr.Christoph Rader, CCR, NCI.

Quantitative RT-PCR

Cells suspended in TRizol® (Ambion, Life Technologies) were kept at -70°C until RNA was extracted following manufacturer's instruction. cDNA was made using random primers (Invitrogen, Life Technologies). Real-time PCR was performed using TaqMan (Life Technologies) gene expression assay probe-primer sets for Tcl1A (ID:Hs00951350_m1) and 18S (ID:HS03003631_g1) and ViiA™ 7 Real-Time PCR System (Applied Biosystems). The expression of target genes relative to internal control gene was calculated using the threshold cycle number (Ct). The relative target gene expression for each condition was normalized to vehicle control and fold change determined using the comparative method ($2^{-\Delta\text{Ct}}$).

Gene expression-microarray analysis

RNA extracted from primary CLL cells that responded to OSU-2S was used for gene expression profiling (GEP) performed in Microarray Shared Resource (MASR) in the OSU Comprehensive Cancer Center. RNA Nano bioanalyzer was used to assess the integrity of the RNA samples. The labeled, fragmented cRNA samples were hybridized to GeneChip Human Genome U133 plus 2.0 (Affymetrix, GPL570).²² Detailed data analysis is described in statistics section. To identify possible molecular and cellular functions affected by OSU-2S, genes changed by >2 fold ($p < 0.0005$) were selected for function and network analyses using Ingenuity pathway analysis (IPA) (<http://www.ingenuity.com>).

Preparation of immunonanoparticle 2A2-ILPs

Liposomes were synthesized with Cholesterol: Egg-PC: PEG-DSPE (molar ratio = 33.5: 65: 1.5) by rapidly injecting lipids dissolved in absolute ethanol into 10 times of phosphate buffered saline (PBS, pH 7.4). 2A2-IgG was reacted with 10x Traut's reagent at room temperature for 1hr at pH 8.0 to yield 2A2-IgG-SH, thus it further reacted with DSPE-PEG-mal at a molar ratio of 1:10 to form micelles at pH 6.8 for 1hr, followed by further incubation with liposomes at the lipid to 2A2-IgG ratio of 2000:1 at 37°C for 1hr. Fluorescent-labeled liposomes (LP-calcein or LP-FAM ODN) were prepared as previously described.²³

Internalization study

The internalization rate of antibodies was determined by measuring and comparing the mean fluorescent intensity of fluorescently-labeled antibodies as described previously²⁴ and outlined in the supplementary information.

Design and preparation of liposomal OSU-2S

The lipids chosen for our study (Cholesterol: Egg-PC: PEG-DSPE; molar ratio = 33.5: 65: 1.5) are widely used to encapsulate small molecule drugs. Characteristics of empty liposome, OSU-2S-LP and 2A2-OSU-2S-ILP are shown in supplementary table 1. The mean diameter by volume of nanoparticles did not change following addition of OSU-2S into the composition. OSU-2S altered the zeta potential of nanoparticles from -4.10 ± 0.34 to 5.41 ± 0.12 mV. By measuring and calculating the amount of OSU-2S before and after dialysis against PBS with LC-MS/MS, the drug entrapment efficiency was $90.09 \pm 2.69\%$ OSU-2S. The step to immobilize 2A2-IgG-PEG-DSPE onto the surface of OSU-2S-LP did

not alter the entrapment efficiency of OSU-2S, and still retained $88.25 \pm 4.81\%$ of OSU-2S in the liposomal form.

E μ -ROR1 transgenic mice generation

The E μ -ROR1 transgenic mice were generated at Genetically Engineered Mouse Modeling facility at the OSU Comprehensive Cancer Center (OSUCCCC) as outlined in the material and methods section in supplementary information. E μ -ROR1 transgenic mouse was crossed with E μ -TCL1 mouse²⁵ (kindly provided by Carlo Croce, OSU) to generate E μ -ROR1-TCL1 double transgenic animals, that were confirmed for the expression of both ROR1 and Tc11 transgenes.

***In-vivo* experiments**

All animal experiments were carried out under protocols approved by OSU Institutional Animal Care and Use Committee (IACUC) modified from our previous report²⁶ and are described in supplementary information.

Statistical analysis of data

All statistical analyses were performed by statisticians in Center for Biostatistics at OSU using SAS 9.3 software (SAS Inc, Cary, NC) as outlined in detail in supplementary information.

RESULTS

OSU-2S mediates PKC dependent cytotoxicity in CLL

OSU-2S mediated potent cytotoxicity in various B cell lines compared to FTY720 (Supplementary Figure S1a). In primary CLL cells OSU-2S caused time, dose {IC₅₀=1.72 μ M, 95% CI 1.59-1.84 μ M} and cell density dependent apoptosis (Figure 1a and Supplementary Figure S1b-d). Potent cytotoxicity in prognostically poor IGVH unmutated (Figure 1b), or chromosome 17p (tumor suppressor p53 locus) deleted (Figure 1c) CLL patient cells, in MEC-2²⁷ and Raji²⁸ (Supplementary Figure S1e-f) cells that are respectively resistant to fludarabine and rituximab, the two widely used CLL therapies, as well as cytotoxicity of fludarabine resistant/refractory patient derived primary CLL cells (Figure 1d) prompted us to evaluate OSU-2S further. OSU-2S induced comparable levels of PP2A activity in CLL cells similar to the parent compound FTY720^{10, 14, 29} (Figure 1e). Since PP2A mediated tumor growth suppression and apoptosis through SHP1 in myeloid leukemia treated with FTY720,¹⁴ we evaluated if OSU-2S affected the SHP1. Interestingly, OSU-2S treatment resulted in increased phospho SHP1^{S591} that was co-immunoprecipitated with PP2A (Supplementary Figure S2a-b) as early as 4hrs post-treatment. While OSU-2S consistently induced increased phospho SHP1^{S591} levels (Figure 2a), there was no change in the levels or enzymatic activity of SHP1 in CLL cells (Supplementary Figure S2c). Consistent with this OSU-2S failed to alter the phosphorylated Src Family Kinase Tyrosine416 (SFK^{Y416}), an auto phosphorylation site and a substrate of SHP1³⁰ (Supplementary Figure S2d). Moreover, comparison of phospho SHP1^{S591} levels (5hr post-treatment) and viability (24hr post-treatment) in OSU-2S treated CLL patient cells showed an inverse correlation (Figure 2b). The OSU-2S-induced phospho Serine 591 is located in C-

terminal nuclear localization sequence of SHP1 protein.^{31, 32} Immunoblotting of phospho SHP1^{S591} and total SHP1 in cytosolic and nuclear extracts of CLL cells treated with OSU-2S revealed abundant phospho SHP1^{S591} in nuclear fraction which was further confirmed by high resolution confocal immunofluorescence microscopy (Supplementary Figure S2e-f). Protein kinase C (PKC) has been shown to co-associate with SHP1 and phosphorylate SHP1^{S591} residue.³³ Consistent with a role for PKC in OSU-2S induced phosphorylation of SHP1^{S591} in CLL cells, bisindolylmaleimide (BIS), an inhibitor of PKC that inhibited PKC activator PMA induced phosphorylation of SHP1^{S591}, also partially prevented OSU-2S induced phosphorylation of SHP1^{S591} (Figure 2a and Supplementary Figure S2g-h) and cytotoxicity (Figure 2c). This was further supported by increase in PKC activity in CLL cells treated with OSU-2S (Figure 2d) and a direct stimulatory effect of OSU-2S on purified PKC in *in-vitro* kinase assays (Figure 2e).

Need for targeted formulation of OSU-2S

Gene expression studies by microarray analysis of RNA isolated from OSU-2S treated CLL cells revealed at least 260 genes that were altered by >2 fold ($p < 0.0005$) (Figure 3a) compared to vehicle treated controls. These genes represent modulators of cellular function and maintenance; development; growth and proliferation; cell death and survival; and cell cycle (Figure 3b). Ingenuity pathway analysis (IPA) of top 40 genes included BCR signaling pathway components, such as PI3K γ , PLC γ , and MAP2K6. Consistent with this, OSU-2S treatment reduced BCR activation of CLL cells stimulated with goat F(ab')₂ against human IgA+IgG+IgM (H+L), as identified with reduced activation marker CD86 (Supplementary Figure S3). TCL1A expression, which is involved in the molecular pathogenesis of CLL³⁴ and identified to be down regulated in response to OSU-2S in the gene expression profile was independently confirmed to be significantly down regulated both at the mRNA and protein levels (Figure 3c) with the corresponding up regulation in cFOS and FRA2 two known inhibitory targets of TCL1A.³⁴ Modulation of ubiquitous phosphatases such as PP2A and SHP1 in unintended target cells and normal B cells where these enzymes are necessary for maintaining cellular functions and homeostasis poses serious limitations in clinical settings.

ROR1 targeted immunonanoparticle formulation of OSU-2S

To target hROR1 surface protein in CLL cells, we considered anti-hROR1 mAb 2A2 (2A2-IgG)³⁵ for immunonanoparticle formulation. The specificity analysis revealed 2A2-IgG stained only leukemic cells from CLL patients but not healthy donor B cells (Supplementary Figure S4a-c). Therefore, targeting ROR1 in CLL patients spares normal B cells, a robust advantage compared to other B cell targeting antibodies such as CD20 or CD19. In addition to specific targeting to leukemic cells, high rate of internalization of 2A2-IgG compared to anti-CD19 or anti-CD37 antibodies was observed in CLL cells (Figure 4a). The confocal microscopic analysis of 2A2-IgG mediated internalization as early as 15 minutes at 37°C in CLL cells is shown in supplementary figure S4d. Selective binding and internalization of 2A2-IgG in CLL cells prompted us to evaluate 2A2-ILPs for targeted delivery of OSU-2S in CLL. 2A2-ILPs loaded with calcein fluorescence molecule co-stained only CD19+ B cells but not CD3+ T cells in PBMC isolated from CLL patients (Supplementary Figure S4e), demonstrating selective binding and uptake of 2A2-ILPs by hROR1+ CLL cells.

Furthermore, normal B cells did not uptake any 2A2-ILP calcein consistent with the lack of expression of hROR1 in normal B cells (Supplementary Figure S4f). CLL cells were dominantly selected by 2A2-ILP calcein while normal B cells or T cells did not uptake 2A2-ILPs as evident from mean fluorescent intensities (Figure 4b). It has been previously reported that 2A2-IgG alone was not able to induce significant direct cytotoxicity, complement dependent cytotoxicity or antibody dependent cellular cytotoxicity in primary CLL cells or hROR1 positive cell lines.³⁵ In contrast to cross-linking effect of anti-CD19 and anti-CD37 ILPs that resulted in direct cytotoxicity,²⁴ evaluation of hROR1+ cell lines, CLL cells or hROR1-ve normal B cells treated with 2A2-IgG, 2A2-IgG cross-linked with α Fc antibody or 2A2-ILPs did not induce cytotoxicity in hROR1+ cell lines or CLL cells (Supplementary Figure S5a-b), thus providing a strong rationale for encapsulation of OSU-2S in 2A2-ILPs for ROR1 targeted delivery in CLL (Supplementary Table 1). CLL cells and normal B cells did not exhibit differences in responses to the treatment with free OSU-2S, OSU-2S-LP or IgG-OSU-2S-ILP (with non-targeting mouse IgG). However, CLL cells but not normal B cells were efficiently killed by hROR1 targeted 2A2-OSU-2S-ILP (Figure 4c-d).

Generation of spontaneous leukemia model for in-vivo evaluation of ROR1 targeted therapy

For *in-vivo* evaluation of hROR1 targeted OSU-2S, we generated E μ -ROR1 transgenic mouse expressing hROR1 exclusively in B cells using a transgenic construct containing the IgH promoter/enhancer elements upstream of hROR1 cDNA²¹ (Supplementary Figure S6a-b). The transgenic line was identified by transgene specific PCR primers and confirmed by Southern blotting. B cell specific hROR1 transgenic protein expression in the splenocytes was confirmed by flow cytometry using hROR1 antibody in combination with B (anti-B220) and T (anti-CD3) cell specific markers (Supplementary Figure S6c-e). 2A2-ILPs loaded with FAM ODN fluorescence molecule showed selective binding of 2A2-ILPs to hROR1+B220+ splenocytes from hROR1 transgenic mouse but not age matched non-transgenic mouse that are hROR1-ve B220+ (Figure 5a-b). Administration of 2A2-OSU-2S-ILP in E μ -ROR1 single transgenic animals resulted in specific depletion of hROR1+ B cells in the peripheral blood (Figure 6a). To recapitulate the human CLL disease with surface hROR1 antigen, the hROR1 transgenic mouse was crossed with a CLL mouse model expressing human TCL1 transgene²⁵ to generate E μ -ROR1-TCL1 double transgenic mice. The development of CLL disease burden in these mice characterized by accumulation surface hROR1+B220+CD5+ leukemic cells in peripheral blood, spleen and bone marrow was confirmed and the splenic hROR1+B220+CD5+ leukemic cells are shown in figure 6b. Further splenocytes from hROR1+ E μ -ROR1-TCL1 (Figure 6c) but not E μ -TCL1 (Figure 6d) mice were significantly killed by 2A2-OSU-2S-ILP in *ex-vivo* cultures. To target leukemic B cells and to avoid off target effects and adverse reaction if any, we used hROR1 targeted 2A2-OSU-2S-ILP in E μ -ROR1-TCL1 double transgenic splenocytes engraftment model for therapy evaluation. Engraftment of B220+CD5+hROR1+ leukemic splenocytes originating from E μ -ROR1-TCL1 double transgenic mice into C57BL/6 mice developed aggressive leukemia (WBC counts $>10 \times 10^3/\mu\text{l}$) with hROR1+B220+CD5+ cells in peripheral blood. These mice were randomly grouped into 2A2-OSU-2S-ILP, IgG-OSU-2S-ILP, OSU-2S-LP or 2A2-Empty-ILP treatment cohorts and dosed 5 days every week for 4

weeks by intra-peritoneal injection of respective drug formulations. The leukemic burden assessed by staining peripheral blood for CD45+B220+CD5+ cells every other week revealed decreased leukemic burden and prolonged median survival (82 days) in 2A2-OSU-2S-ILP treated animals compared to 2A2-Empty-ILP group (22 days) (Figure 6e-f).

DISCUSSION

Chemotherapeutics remains a choice of treatment for several malignant diseases. Here we describe a CLL tumor antigen ROR1 targeted delivery of immunonanoparticle carrying a novel non-immunosuppressive phosphatase activating FTY720 derivative OSU-2S with potent cytotoxicity against leukemic B cells sparing normal B cells. These studies have focused on rational preclinical development of FTY720 derivative OSU-2S, its *in-vitro* and *in-vivo* mechanistic and therapeutic evaluations using novel targeted immunoliposomal delivery formulations and humanized mouse models of leukemia developed for this purpose. Despite promising *in-vitro* and *in-vivo* activity of FTY720 against a variety of solid tumors and heme-malignancies,^{10, 11, 13-15} our attempts to repurpose FTY720, a clinically approved drug for relapsing multiple sclerosis, for oncology indications were hindered by its immunosuppressive property involving T cell homing to lymph nodes. Hence, OSU-2S was rederived to distinguish it from the parent FTY720 compound in 3 aspects:¹⁶ i) Unlike FTY720, OSU-2S is not phosphorylated by sphingosine kinase-2 in *in-vitro* systems; ii) synthetic phosphorylated OSU-2S failed to internalize S1PRs in cell lines expressing S1PR but is internalized when treated with FTY720 or phosphorylated FTY720; iii) administration of OSU-2S to mice did not cause drastic peripheral blood T cell count reduction in contrast to FTY720 which engender lymph node homing of T cells (Supplementary Figure S7).

Congruent with FTY720, OSU-2S activated PP2A in CLL cells. Interestingly, OSU-2S induced association of PP2A and phospho SHP1^{S591} in CLL cells. Since SHP1 dependent PP2A mediated tumor growth suppression and apoptosis by reduced BCR/ABL expression and activity was established in CML,⁵ we sought to see if OSU-2S affected the enzyme activity of SHP1. While OSU-2S failed to modulate the SHP1 phosphatase activity, it induced phosphorylation of SHP1^{S591} at the putative PKC substrate motif (K/RXS*XX/R)³⁶ on SHP1. Consistent with our speculation on the role for PKC in OSU-2S induced phosphorylation, concentrations of PKC inhibitor BIS that reduced the PMA induced PKC activity also inhibited OSU-2S induced phosphorylation of SHP1^{S591} in CLL cells. This was also supported by increase in PKC activity in CLL cells. More importantly OSU-2S had a direct stimulatory effect on purified PKC in our *in-vitro* kinase assays. These finding were consistent with literature evidence suggesting PKC as a relevant phosphorylating kinase of SHP1^{S591} residue.^{33, 37} Experimental conditions reducing the phosphorylation of SHP1 also significantly prevented the cytotoxicity of OSU-2S implying the potential role of SHP1 in OSU-2S mediated apoptosis. The expression of SHP1 is drastically lowered in many leukemias and lymphomas due to DNA promoter hyper methylation implying negative role of SHP-1 in development of leukemia/lymphoma.³⁸ Moreover, restoration of SHP1 in leukemia having low expression or devoid of SHP1 resulted in sensitization or direct death of tumor cells.³⁹⁻⁴¹ Further, it was recently shown that SHP1 is directly involved in promoting death signals in CLL cells after ligation of tetraspanin CD37.⁴² Our preliminary experiments indicated that cell lines expressing low levels of SHP1 are less sensitive to

OSU-2S and when reconstituted with SHP1 became sensitive to OSU-2S only when SHP1^{S591} was phosphorylated, altogether confirming the importance of SHP1^{S591} phosphorylation in OSU-2S cytotoxicity. We did not find change in enzyme activity of phosphorylated SHP1^{S591} in CLL. This was supported by established cell type specific roles of S591 including increase or decrease in enzyme activity and nuclear localization signals. Phorbol ester had been shown to induce SHP1 activation with serine phosphorylation in HL-60 pro-myelocytic cells accompanied by growth arrest.⁴³ However, in our study we saw no change in the enzyme activity, but nuclear localization of phospho SHP1^{S591} and cell death after treatment with OSU-2S. It is possible that nuclear translocated SHP1 might act on nuclear substrates or interact with other nuclear proteins regulating transcription to bring about cell death in our model.

The novel strategy to enhance the efficiency and specificity of OSU-2S drug delivery in CLL using hROR1 targeting ILP has promising clinical implications. ROR1 identified as oncofetal protein is expressed during embryogenesis and in some malignancies including CLL cells but absent in mature normal B cells.^{17, 18, 44} Recent discovery of ROR1 expression in B cell precursor, hematogones may indicate toxic potential of 2A2-OSU-2S-ILP on these population.⁴⁵ But it should be noted that mature normal B cells or CD34+ precursors lack ROR1 and hematogones have low ROR1 expression and occur in very low frequencies compared to CLL. Furthermore, recent reports using different ROR1 antibodies indicate that ROR1 expression and its phosphorylation are directly correlated with disease severity in CLL and can enhance leukemogenesis in CLL.⁴⁶⁻⁴⁸ Thus, the unique expression pattern of ROR1 in CLL cells makes it an attractive target for therapy. This has led to development of various ROR1 targeted therapeutics including anti-ROR1 antibodies raised against different epitopes of ROR1 and have different cytotoxic potential.^{35, 48-51} Moreover, Fc domain engineering of anti-ROR1 antibodies are undertaken to enhance the effector functions like antibody dependent cell-mediated cytotoxicity(ADCC), complement dependent cytotoxicity(CDC) or properties of antibody-drug conjugate(ADC). The recently reported ROR1 directed chimeric antigenic receptor (ROR1-CAR) designed using 2A2-ScFv on T cell demonstrating potent anti-leukemic property against ROR1+ cells is noteworthy.⁵² Consistent with previous reports, our results demonstrated that anti-hROR1 mAb, 2A2-IgG selectively binds to CLL cells, is internalized efficiently, and does not induce cytotoxicity as a single reagent or in the presence of antibody or liposomal cross linker.³⁵ Further, due to the limited cell surface density, hROR1 is considered preferably a target for armed rather than naked mAbs, though other anti-hROR1 mAbs inducing apoptosis in CLL have been reported.^{48, 50} It is conceivable that incorporation of cytotoxic anti-hROR1 mAbs as well as the use of human or humanized anti-hROR1 mAbs in ILP formulations has potential clinical utility.

The liposomal packaging of OSU-2S reduced the risk of other cells being exposed to the drug, however the minimal effect of OSU-2S-LP on normal B cell (Figure 4c & Supplementary Figure S8) may be due to prolonged exposure of these cells to lingering OSU-2S-LP particles which could have caused the particles to be fusing to normal B cells. Moreover, in targeted therapy 2A2-OSU-2S-ILP particles would be consumed by malignant cells and induce cytotoxicity and therefore fewer particles would be available for normal cells to get exposed and additional dosing can be used to eliminate the malignant cells if

needed. Additionally, the concentration of targeted particles could be lowered and optimized in a range that can be toxic only to malignant cells for which extensive pharmacokinetic/ pharmacodynamic studies are needed and to confirm the integrity of the liposomes. Interestingly, 2A2-OSU-2S-ILP promoted comparable levels of cytotoxicity as CD20-OSU-2S-ILP in ROR1+CD20+ CLL cells (Supplementary Figure S9). Moreover, OSU-2S did not change the expression pattern of ROR1 in CLL which would support that OSU-2S neither lowers the target for 2A2-OSU-2S-ILP nor it enhances the ROR1 expression mediated survival in CLL cells (Supplementary Table 2). While cell surface CD19 did not change after OSU-2S treatment in CLL, there was mild decrease in CD20 surface protein although statistically insignificant (Supplementary Table 3). These findings would support that OSU-2S does not alter the targets CD19 or CD20 which can be used for salvage therapy in CLL.

We have tested the activity OSU-2S as a single agent in a novel CLL mouse model (Supplementary Figure S10). In order to test the drug in a clinically relevant setting and to avoid drug adverse reaction, if any, we decided to deliver OSU-2S using an ILP formulation that could selectively target leukemic B cells in a mouse model of CLL. To accomplish this, we generated E μ -ROR1 transgenic mice and crossed with the E μ -TCL1 leukemia mouse model. These double transgenic mice exhibited CLL like disease later in life (>9 months) with additional expression of hROR1 in B220+CD5+ leukemic cells. Since these mice express hROR1 and to conduct an *in-vivo* study in a uniform leukemic model, we adapted the leukemic cells suitable for allograft model by passaging the leukemic clones in wild type background mice. By confirming the tumor burden in peripheral blood after engrafting hROR1+ leukemic splenocytes in experimental animals, we then started treatment with OSU-2S ILP targeting hROR1 which resulted in cytotoxicity specific for leukemic cells. The OSU-2S immunonanoparticle formulation that selectively targets hROR1+ leukemic CLL but not normal B cells *in-vitro* and in a humanized CLL mouse model rendering therapeutic benefit adds credence to the approach described here and can be extended to other therapeutic agents and ROR1+ malignancies including mantle cell lymphoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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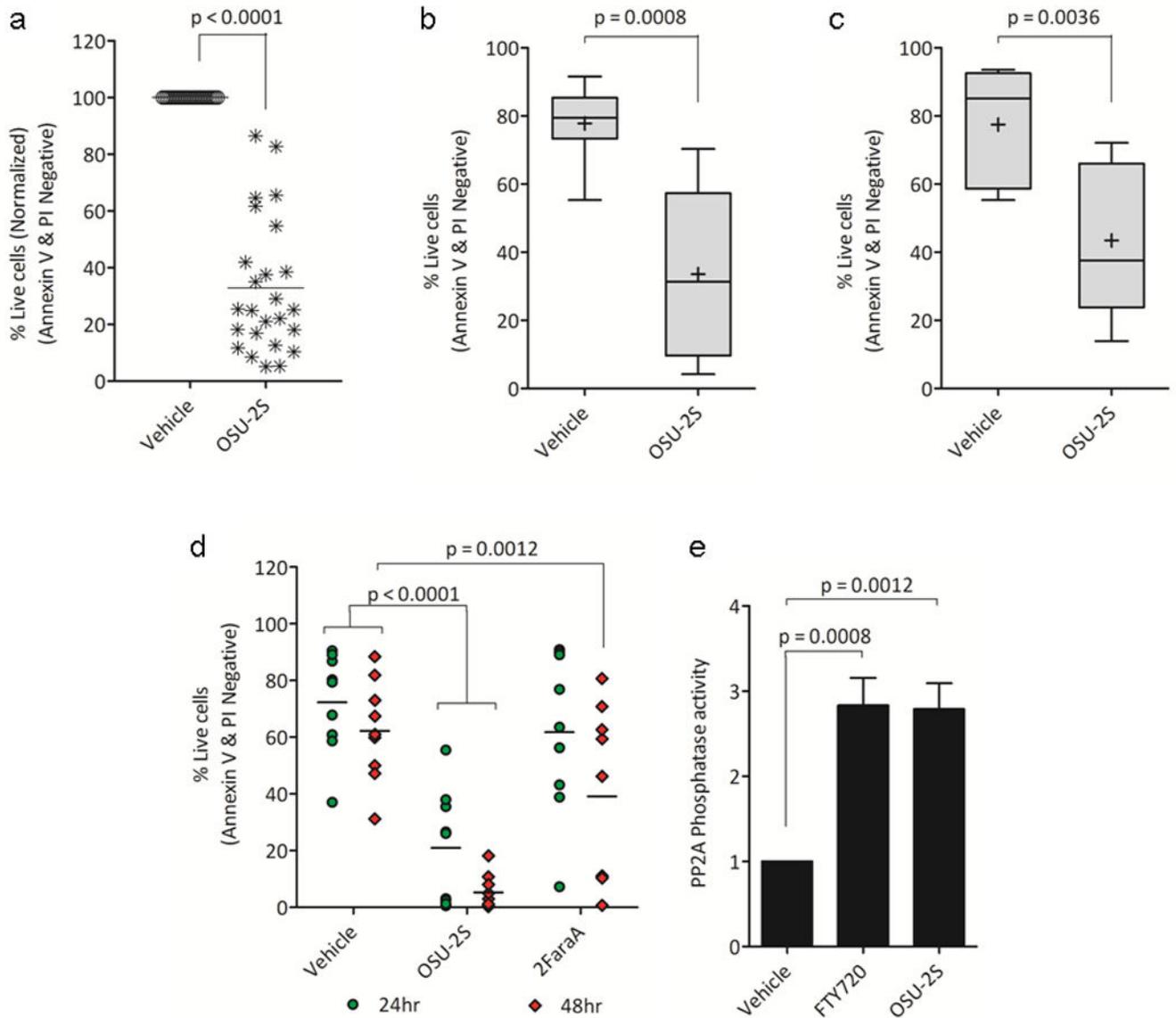


Figure 1.

OSU-2S mediates cytotoxicity in CLL primary cells. **(a-c)** Cytotoxic effects of OSU-2S in CLL. Freshly isolated CD19⁺ CLL primary cells (1×10^6 /ml) were treated with OSU-2S (2 μ M) and viability was assessed 24 hours later. **(a)** Mixed CLL samples (N=25). **(B-C)** Activity of OSU-2S in prognostically poor CLL patient derived primary cells **(b)** IGVH unmutated (N=8) and **(c)** chromosome 17p deleted (N=5) CLL cells. **(d)** Comparison of OSU-2S and Fludarabine (2FaraA) cytotoxicity in CLL. CLL cells (10×10^6 /ml) were treated with OUS-2S(8 μ M) or 2FaraA(10 μ M) and viability was measured at indicated time points (N=9). **(e)** OSU-2S activates PP2A. CLL cells treated with OSU-2S or FTY720 were used for an *in-vitro* PP2A phosphatase enzyme assay. Values were normalized to protein levels and vehicle treated control; bars represent SD (N=3).

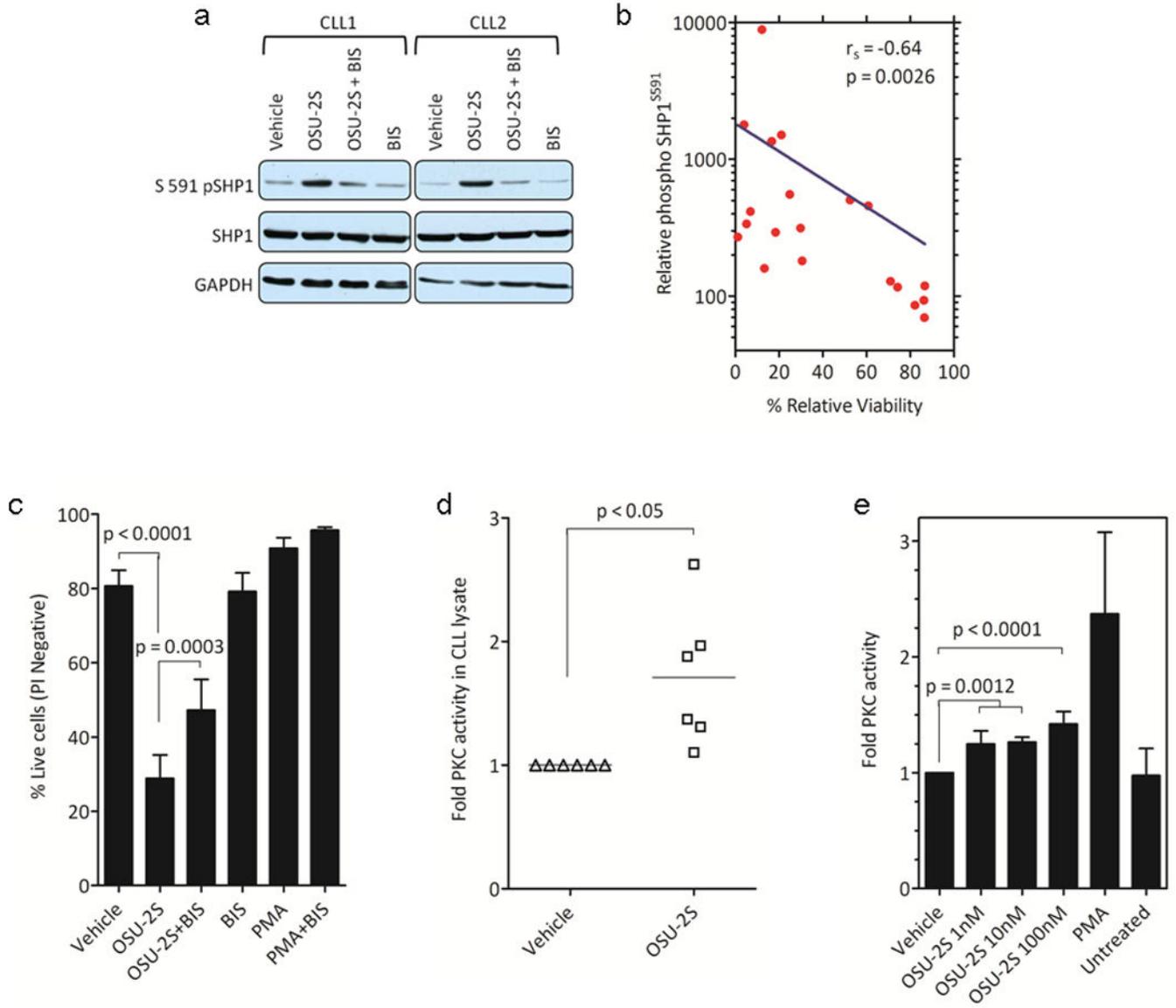


Figure 2. OSU-2S mediates PKC dependent phosphorylation of SHP1^{S591} in CLL. (a) OSU-2S induces PKC dependent phosphorylation of SHP1^{S591} in CLL. CLL cells treated with OSU-2S(5 hours) were immunoblotted for phospho SHP1^{S591}. Bisindolylmaleimide (BIS) was used to inhibit PKC. (b) Inverse correlation between phospho SHP1^{S591} and viability. Spearman rank-order correlation was calculated for phospho SHP1^{S591} induced after 5 hour by immunoblotting and percent change in the viability at 24 hour time point in response to OSU-2S treatment (N=20). (c) PKC inhibitor BIS reduces phospho SHP1^{S591} and rescues OSU-2S cytotoxicity. Viability was done at 24 hour time point by PI staining on CLL cells treated with Vehicle or OSU-2S in the presence or absence of PKC inhibitor BIS(2μM) (N=8); bars represent SE. PMA is used as control for BIS. (D-E) OSU-2S activates PKC. (d) PKC activity was assessed in CLL cell lysates collected 4hr after treatment with vehicle or

8 μ M OSU-2S, (N=6). (e) Addition of OSU-2S to purified rat PKC had a direct stimulatory effect. PMA is used as positive control (N=3).

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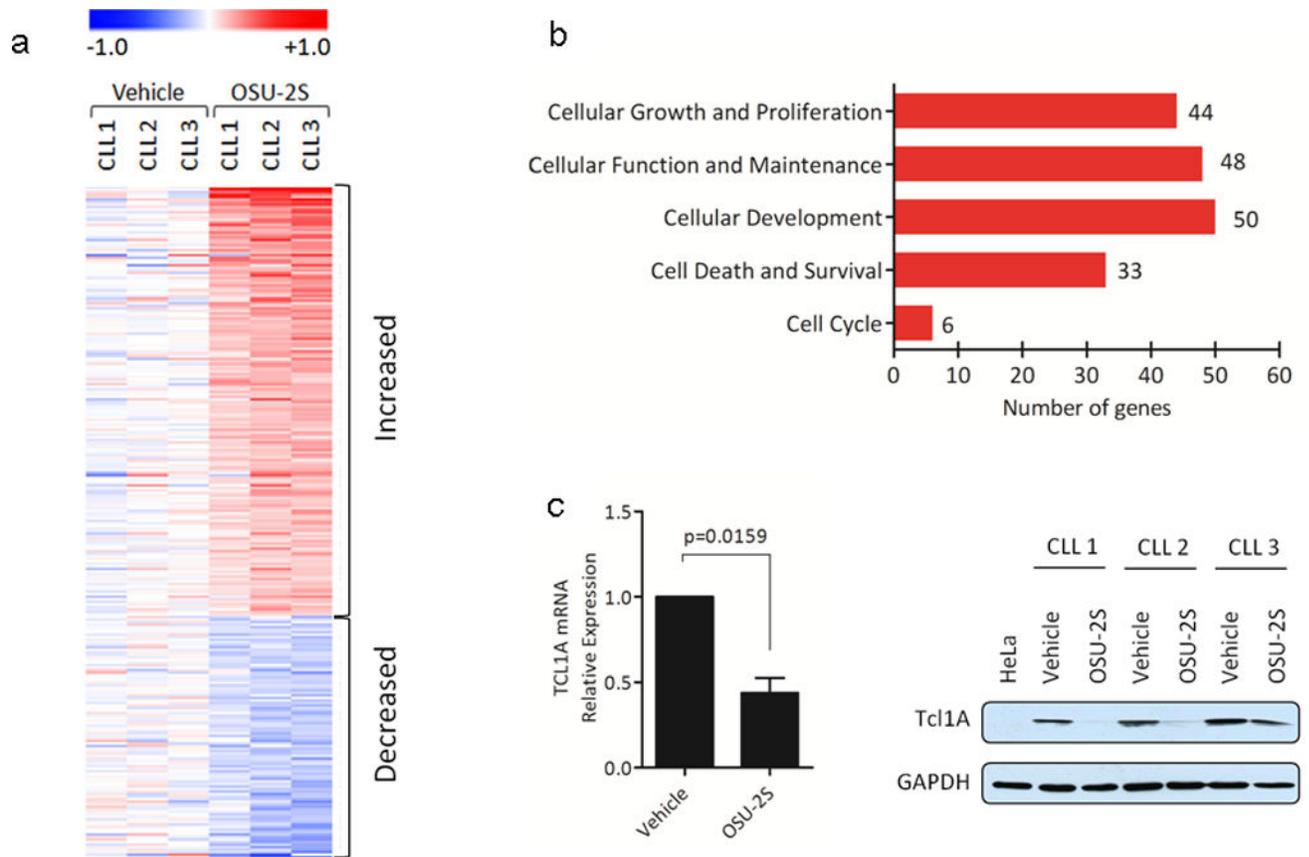


Figure 3.

Gene expression analysis of CLL cells treated with OSU-2S. **(a)** Heat map of 266 differentially expressed genes in OSU-2S treated CLL. RNA was isolated from CLL cells (N=3) treated with vehicle or OSU-2S(8 μ M) at 16 hours and subjected to microarray analyses using GeneChip Human Genome U133 plus 2.0 (Affymetrix, GPL570). After statistical analyses, genes that changed by ≥ 2 fold and $p < 0.0005$ in response to OSU-2S treatment were used for constructing a heat map. **(b)** Top five molecular and cellular functions revealed through Ingenuity Pathway analysis (IPA) of genes modulated by OSU-2S in CLL. **(c)** OSU-2S down modulates TCL1A. RNA was extracted from vehicle or OSU-2S treated (16hr) CLL patients cells (N=7) and TCL1A expression relative to 18S was determined by real-time RT-PCR analysis and values are normalized to vehicle control; bars represent SD. Immunoblot of TCL1A in CLL cells treated with vehicle or OSU-2S for 24 hours is also shown.

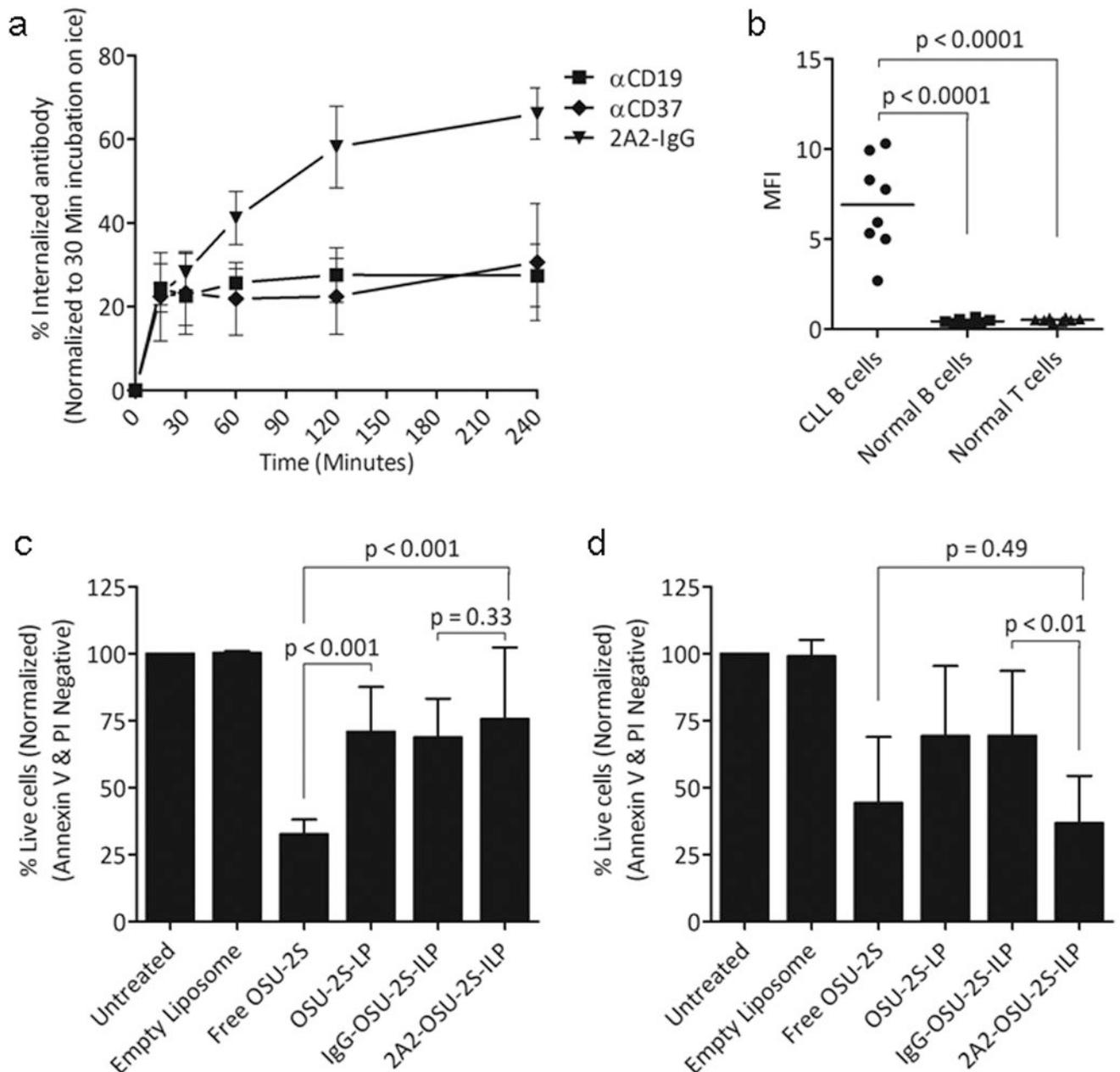


Figure 4.

Immunonanoparticle 2A2-ILPs targeting ROR1+ CLL cells for OSU-2S drug delivery. (a) Internalization curve for 2A2-IgG bound ROR1 in CLL cells. Internalization involving time-dependent increase in mean fluorescent intensity (MFI) of labeled antibody on CLL cells treated with 2A2-IgG, α CD19 or α CD37 is shown. Bars represent mean \pm SD, $P < 0.001$ ($N=7$). (b) Internalization of 2A2-ILP. MFI showing the binding and uptake of 2A2-ILP calcein by CLL cells, normal B cells and T cells ($N=8$, $P < 0.0001$). (c-d) 2A2-OSU-2S-ILP mediates specific cytotoxicity in CLL cells. 1×10^6 normal B cells from healthy donors or CLL cells were incubated with different formulation at $5 \mu\text{M}$ of OSU-2S and $0.1 \mu\text{g/ml}$ mAbs

for 24hr before viability was analyzed by flow cytometry (mean \pm SEM). **(c)** No difference in cytotoxicity between 2A2-OSU-2S-ILP and IgG-OSU-2S-ILP formulations was found for normal B cells (N=4). **(d)** 2A2-OSU-2S-ILP significantly induced improved cytotoxicity in CLL cells compared to non-targeting IgG-OSU-2S-ILP (N=8, $P < 0.01$).

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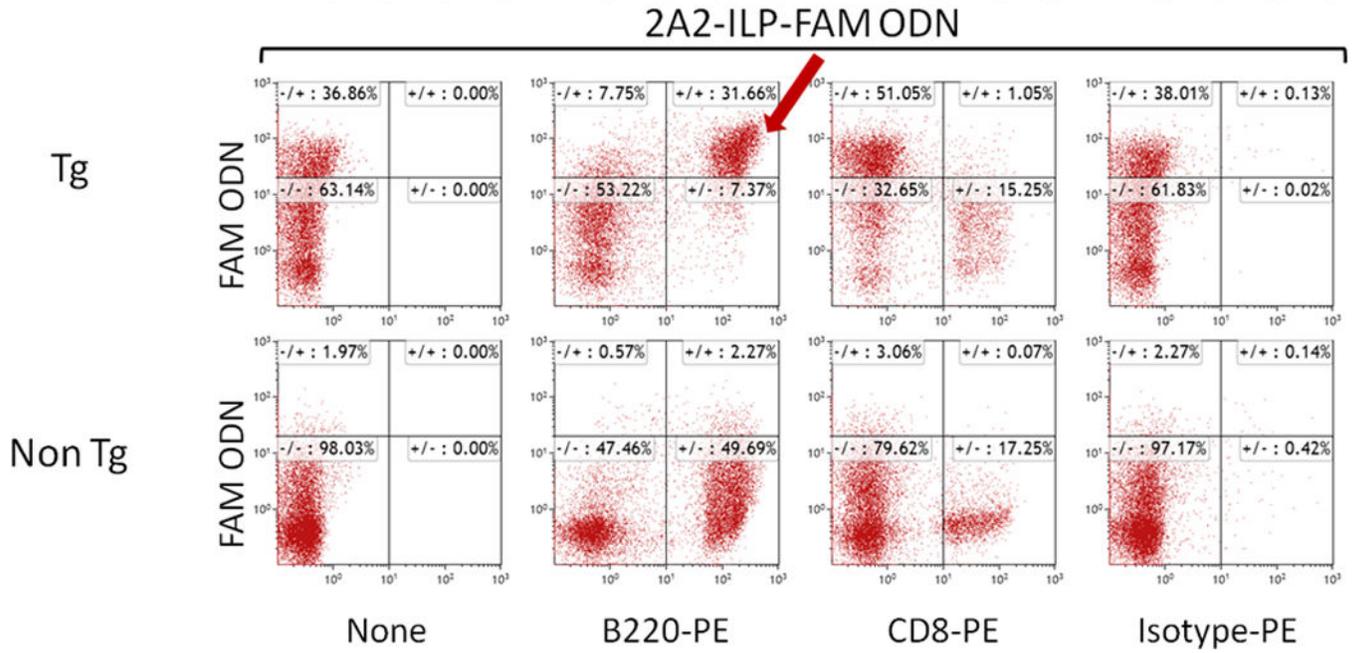


Figure 5. Immunonanoparticle 2A2-ILPs selectively bind to hROR1+ B cells from Eμ-ROR1 transgenic mice. Splenocytes (1×10^6) from Eμ-ROR1 transgenic(Tg) or age matched non-transgenic(Non Tg) mice were treated with 2A2-ILP FAM ODN for 30 minutes at 37°C, washed and stained with mouse B220-PE or CD8-PE. Flowcytometric analysis revealed FAM ODN signal only in B220+ Eμ-ROR1 transgenic mice but not to B cells from non-transgenic mice.

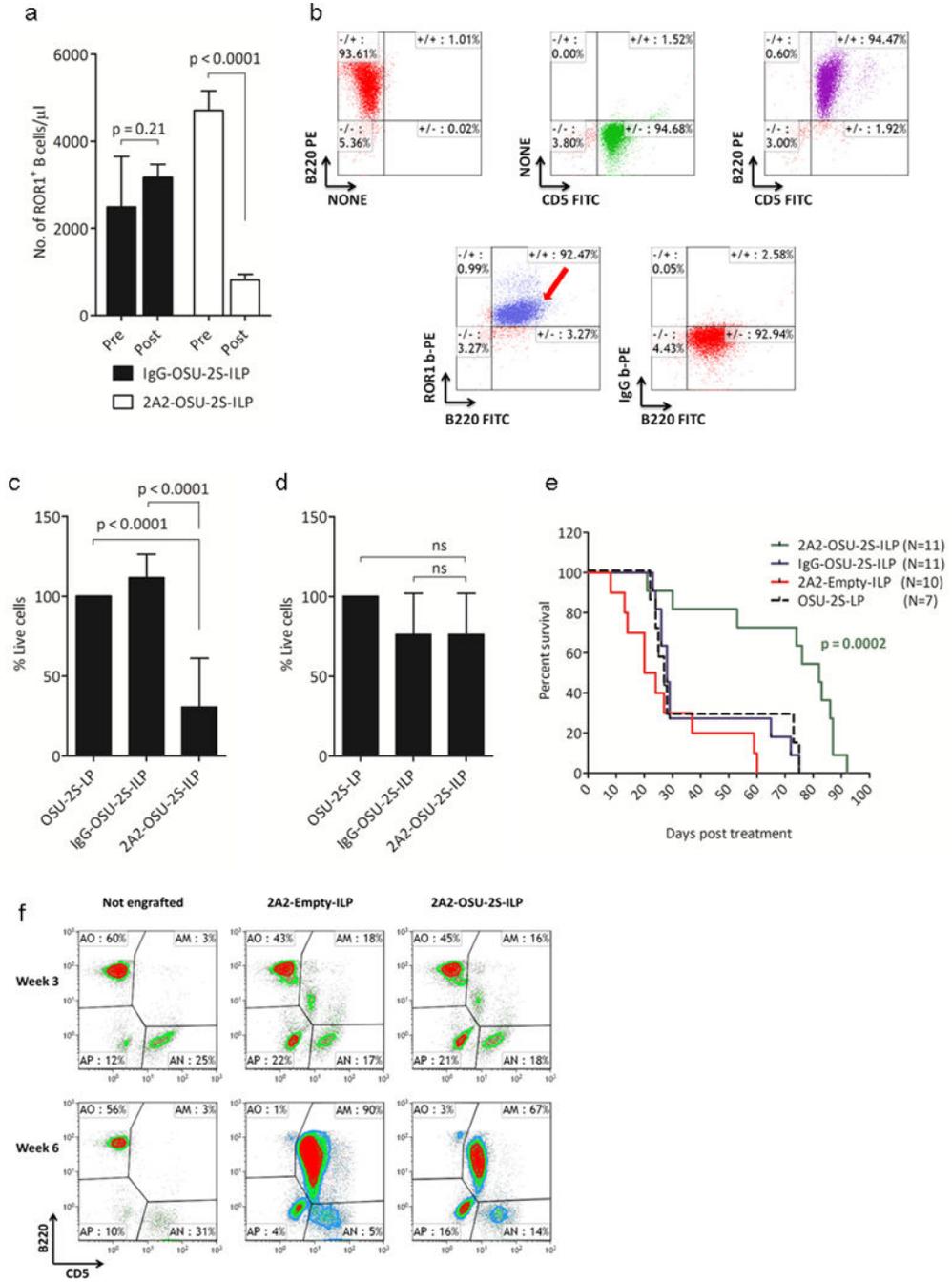


Figure 6. ROR1 targeted delivery of OSU-2S prolongs survival in hROR1+ CLL mouse model. (a) Specific depletion of ROR1+ B cells in peripheral blood of Eμ-ROR1 single transgenic animals after three doses of 2A2-OSU-2S-ILP intravenous treatment (10mg/kg every other day). (b) Flowcytometric dot plot of hROR1+ splenic cells from Eμ-ROR1-TCL1 animal with leukemia. (c-d) Comparison of specific cytotoxicity of 2A2-OSU-2S-ILP in Eμ-ROR1-TCL1(N=8) (c) and Eμ-TCL1(N=6) (d) mice derived splenocytes cultured ex-vivo with different ILPs(5μg/ml) and viability was assessed after 16hr by flowcytometry. (e-f) ROR1

targeted delivery of OSU-2S prolongs survival in hROR1+ CLL mouse model. (e) Survival curve of E μ -ROR1-TCL1 splenocytes engrafted mice treated with 2A2-OSU-2S-ILP(N=11); IgG-OSU-2S-ILP(N=11), OSU-2S-LP(N=7) or 2A2-Empty-ILP(N=10). (f) Peripheral blood analysis of leukemic burden in the engrafted mice by flowcytometry.

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