ROR1 targeted delivery of OSU-2S, a non-immunosuppressive FTY720 derivative exerts potent cytotoxicity in mantle cell lymphoma in-vitro and in-vivo.

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Abstract:

Mantle cell lymphoma (MCL) remains incurable despite numerous therapeutic advances. OSU-2S, a novel non-immunosuppressive FTY720 (Fingolimod) derivative, exhibits potent cytotoxicity in MCL cell lines and primary cells. OSU-2S increased the surface expression of CD74, a therapeutic antibody target in MCL cells. OSU-2S in combination with anti-CD74 antibody, milatuzumab, enhanced cytotoxicity in MCL. Moreover, MCL tumor antigen ROR1 targeted immunonanoparticle carrying OSU-2S (2A2-OSU-2S-ILP) mediated selective cytotoxicity of MCL *in-vitro*, and activity in xenografted mouse model of MCL *in-vivo*. The newly developed OSU-2S delivery using ROR1-directed immunonanoparticles provide selective targeting of OSU-2S to MCL and other ROR1+ malignancies sparing normal B cells.

Key words:

MCL, FTY720, OSU-2S, CD74, ROR1, targeted delivery

Introduction:

MCL is an uncommon non-Hodgkin's lymphoma with limited treatment options[1]. We have previously shown the pre-clinical effect of FTY720, a sphingosine analogue, in MCL. Treatment of MCL cells with FTY720 resulted in potent cytotoxicity with concomitant decrease in cyclin D1 and phospho AKT proteins in MCL cell lines and primary MCL patient samples[2, 3]. Furthermore, FTY720 induced cell surface expression of CD74, a therapeutic target for milatuzumab, an anti-CD74 antibody through blockage of autophagic pathway[3]. Though these findings supported the combination of FTY720 and milatuzumab, the T-cell sequestering immunosuppressive nature of FTY720 diminished the enthusiasm for its clinical development. Hence, we developed a FTY720 derivative OSU-2S[4], through structure modification so that it does not get phosphorylated or bind to sphingosine-1-phosphate-receptor, a property that is essential for T cell sequestration and immunosuppression by FTY720. Using *in-vivo* models we have previously reported that unlike FTY720, OSU-2S does not alter T cell trafficking[4, 5]. Anticipated off-target effects of OSU-2S prompted us to develop targeted delivery formulation directed towards ROR1, a receptor tyrosine kinase receptor expressed on the surface of MCL tumor cells but not normal B cells[6]. Herein, we validated the potent direct cytotoxicity of OSU-2S in MCL cells and evaluate a tumor directed lipid based nanoparticle formulation of OSU-2S designed to selectively deliver to ROR1+ MCL cells.

Materials and Methods:

Cells: Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MCL primary cells were obtained from patients' blood or lymph node biopsy following

providing written informed consent under a protocol approved by the institutional review board (IRB). Mononuclear cells were isolated by ficoll density gradient centrifugation (Ficoll-Paque Plus; Amershan Biosciences, Piscataway, NJ, USA) and cultured in RPMI 1640 media (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA); 2 mM L-glutamine and penicillin (100 U/mL); streptomycin (100 µg/mL) (Gibco) at 37°C with 5% CO₂ aeration.

Cell viability and surface markers: Apoptosis was assessed by annexin-V-FITC and propidium iodide as described previously[5]. Cell proliferation was measured by CellTiter96 MTS proliferation assay (Promega, Madison, WI, USA) according to manufacturer's instruction. Cells were stained for surface markers by standard methods and read in FC-500 flow cytometer (Beckman-Coulter, Brea, CA, USA). Live dead stain (Invitrogen, Life Technologies) was used in combination with surface markers for gating live cells in flow cytometric experiments. Flow cytometric data were analyzed using Kaluza software (Beckman-Coulter).

Chemicals and reagents: OSU-2S was synthesized at the Ohio State University (OSU) Medicinal Chemistry Shared Resource as described previously[4]. FTY720 was purchased from ChemieTek (Indianapolis, IN). ROR1 (R&D Systems, Minneapolis, MN, USA) CD19 and CD74 (BD Bioscience, San Jose, CA, USA) antibodies were used for flow cytometric analyses. Mouse monoclonal 2A2-IgG against human ROR1 was described previously[7]. Milatuzumab was obtained from Immunomedics.

2A2-OSU-2S-ILP synthesis: Immunonanoparticles were synthesized as described previously[5]. Briefly, Cholesterol: Egg-PC: PEG-DSPE (molar ratio 33.5: 65: 1.5) were used to prepare liposomes by forcedly injecting the above mixture prepared in absolute ethanol into 10X volume

phosphate buffered saline(PBS) pH 7.4. Traut's reagent was used to reduce 2A2-IgG at room temperature to form 2A2-IgG-SH which was further incubated with DSPE PEG-mal pH 6.8 (molar ratio 1:10) to form 2A2-IgG micelles. The liposomes and 2A2-IgG micelles were combined (lipid : 2A2-IgG molar ratio 2000:1) and incubated at 37°C. OSU-2S was encapsulated into liposomes by ethanol injection method before tethering IgG on the liposomal surface. OSU-2S was dissolved in absolute ethanol at 10µM before addition of lipids (Cholesterol, Egg-PC and PEG-DSPE) and the fully mixed OSU-2S and lipids (1:20) were forcedly injected into 10X volume PBS pH 7.4 to form OSU-2S-LP.

In-vivo experiments: Animal experiments were done under the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of OSU. Six week-old female NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) mice (Jackson Laboratories, Bar Harbor, Maine, USA) were engrafted with 4 million Mino cells in PBS in the left flank region by subcutaneous injection. All animals were monitored for signs of disease, tumor size by digital calipers and other exclusion criteria including ulcerated tumor, extended anorexia or other diseases. Treatment began 2 weeks after engraftment when palpable tumors were noted. Mice were randomly grouped and treated with 2A2-OSU-2S-ILP; IgG-OSU-2S-ILP or 2A2-Empty-ILP at the dose of 10 mg/kg of OSU-2S daily for 10 days by intra-peritoneal injections. All animals were sacrificed 25 days post engraftment and the tumor mass was harvested and weighed by a person blinded to the study.

Statistical analysis: Statistical analyses were performed by statisticians in the Center for Biostatistics at OSU using SAS 9.3 software (SAS, Cary, NC, USA). For data with repeated measures, mixed effect models were used considering observational dependencies across subjects. Matched samples were compared by paired t-tests. Analysis of variance(ANOVA) was

used to compare means of multiple independent groups. Multiplicities were adjusted by Holm's method.

Results and Discussion:

OSU-2S had profound anti-mitochondrial activity and cytotoxicity in JeKo[8], Mino[9] and other MCL cell lines (Fig 1A-B & S.Fig 1A) and cytotoxicity in MCL patient-derived primary cells (Fig 1C). Treatment of JeKo and Mino cells with OSU-2S caused processing of the caspase-3 substrate PARP as detected by immunoblotting (not shown). Interestingly, compared to the vehicle control, OSU-2S induced surface expression of CD74, the target of a therapeutic antibody milatuzumab in MCL cells (Fig 1D). Evaluation of OSU-2S and milatuzumab combination therapy in primary MCL cells revealed enhanced cytotoxicity compared to either of the agents alone (Fig 1E). Similarly, induction of surface CD74 by OSU-2S and enhanced cytotoxicity with milatuzumab was also confirmed in JeKo and Mino cells (not shown).

Tumor directed delivery of cytotoxic cargo offers the potential to further enhance the therapeutic index of cancer therapeutics such as OSU-2S. Restricted expression of receptor tyrosine kinase ROR1 in several B cell malignancies including MCL, CLL and subset of pediatric ALL has been reported[5, 6, 10-12]. Consistent with this, JeKo, Mino (Fig 2A) and primary MCL cells but not normal B cells expressed ROR1 (Fig 2B & S.Fig 1B). To deliver the OSU-2S selectively to MCL cells, we developed a lipid-based OSU-2S nanoparticle (OSU-2S-LP) tethered with an anti-ROR1 mouse monoclonal antibody (2A2). This immunonanoparticle formulation is designated as 2A2-OSU-2S-ILP. Binding of 2A2 antibody leading to ROR1 internalization has been reported in JeKo and Mino cells which would facilitate our drug delivery strategy in those cells[5, 7]. Restricted expression of ROR1 on tumor B cells but not normal B cells will facilitate

selective delivery of the drug to lymphoma cells through ROR1 thus preventing normal B cell from cytotoxicity and therefore protecting their functional integrity. Testing of immunonanoparticle formulation on JeKo, Mino cell lines (Fig 2C) and primary MCL cells (Fig 2D) showed selective cytotoxicity of 2A2-OSU-2S-ILP in MCL compared to other controls, including non-targeted formulation (OSU-2S-LP), non-specific antibody (IgG-OSU-2S-ILP) or empty targeted formulation (2A2-Empty-ILP). Moreover, we found that OSU-2S did not alter ROR1 expression on MCL cells(not shown) and therefore the target for 2A2-OSU-2S-ILP is not compromised. It is important to note that 2A2 or 2A2+crosslinker or 2A2-Empty-ILP has no cytotoxic effect on ROR1 expressing cells. We then evaluated 2A2-OSU-2S-ILP in a preclinical xenograft mouse model of MCL using Mino cell line as described recently[13]. *In-vivo* treatment of 2A2-OSU-2S-ILP in xenografted mice revealed significantly diminished tumor growth compared to the controls (Fig 2E & S.Fig 1C). While IgG-OSU-2S-ILP had some *in-vivo* activity presumably due to generalized effect of OSU-2S, it has no target mediated disposition unlike 2A2-OSU-2S-ILP which has higher affinity for ROR1+ malignant cells and potential disposition. Further, a nonspecific IgG-OSU-2S-ILP would not be used in clinical subjects as extended pharmacokinetic duration of the particles *in-vivo* may become non-specific. Overall, these findings describe the novel OSU-2S molecule as a successor of FTY720 that is active against MCL. OSU-2S enhanced CD74 expression can be exploited in combination therapy with milatuzumab which is in human clinical trials. Importantly, the tumor antigen ROR1 directed delivery system for increasing the payload selectively to the cancer cells thus obviating the exposure of chemotherapeutics and targeted therapeutics to other unintended target cells has broad implications in diverse ROR1+ B cell malignancies including MCL, CLL and B-ALL.

Conflict of interest: A patent has been filed and awarded to OSU for the use of OSU-2S to treat hematologic malignancies. The authors declare no additional competing financial interests.

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Author contributions: R.M. planned, designed and performed experiments, analyzed data, wrote the drafts of the paper. C.C synthesized immunonanoparticles. F.F. assisted with the experiments. R.Y. synthesized OSU-2S. X.M. performed the statistical analysis. S.B., C.R., M.A.P., C.S.C., R.J.L., J.C.B. and L.J.L. provided necessary reagents, supported components of the research. R.B. contributed to the MCL patient care, patient sample acquisition and characterization, translation insight to the experimental designs. N.M oversaw the planning and study design, sought funding, reviewed drafts of the manuscript and approved the final version for submission.

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Figure legends:**Figure 1: Activity of OSU-2S in MCL cell lines and primary cells.**

(A) Comparison of OSU-2S and FTY720 in JeKo and Mino cell lines. Cells ($1 \times 10^6/\text{mL}$) were treated with indicated concentrations of drug and activity was assessed after 24hr by MTS assay (N=3). The trend for each of the drug was significant in both the cell lines ($p < 0.0001$) and OSU-2S was more potent than FTY720 as estimated by the slope ($p < 0.0001$). (B) Cytotoxicity of OSU-2S ($5 \mu\text{M}$) in MCL cell lines after 24hr (N=3) as measured by Annexin/PI staining. Bars represent mean with SE. (C) Cytotoxicity of OSU-2S in primary MCL cells (N=8) obtained by ficoll gradient centrifugation of patient blood or lymph node cells and cultured ($2 \times 10^6/\text{mL}$) with $4 \mu\text{M}$ of OSU-2S for 24hr. (D) CD74 induction by OSU-2S in patient derived primary MCL cells. Primary MCL cells ($2 \times 10^6/\text{mL}$) were treated with $4 \mu\text{M}$ of OSU-2S for 24hr and stained for cell surface CD74. CD74 increased in response to OSU-2S, but no change in CD20 which was used as control (representative of three independent experiments). Mean fluorescence intensity (MFI) of CD74 and isotype control for different treatments are shown. (E) Combination therapeutics with OSU-2S and milatuzumab in primary MCL cells has enhanced effect. Primary MCL cells (N=4, $2 \times 10^6/\text{mL}$) were cultured with $4 \mu\text{M}$ of OSU-2S and milatuzumab ($5 \mu\text{g/mL}$) with cross linker (αFc) and viability was analyzed at 24hr by flow cytometry. Bars represent mean with SE.

Figure 2: ROR1 expression and ROR1 targeted delivery of OSU-2S in MCL.

(A-B) ROR1 is expressed in MCL cell surface. ROR1 expression in JeKo, Mino (A) and patient derived primary MCL cells (B) assessed by flow cytometry using goat polyclonal anti-human ROR1-Alexa-fluor-488 antibody. MFI is shown for ROR1 stained, unstained and isotype

controls. **(C-D)** ROR1 targeted delivery of OSU-2S using immunonanoparticle 2A2-OSU-2S-ILP is selectively cytotoxic in JeKo, Mino cells **(C)** and patient derived primary MCL cells **(D)**. JeKo and Mino cells (1×10^6 /mL) (N=3) and patient derived primary MCL cells (2×10^6 /mL) (N=8) were cultured with $2 \mu\text{g/mL}$ of different immunonanoparticle formulations for 24 hours and viability was assessed by flow cytometry. Bars represent mean with SE. **(E)** ROR1 targeted nanoparticle formulation of OSU-2S is active in MCL xenograft model. Mino cells were engrafted into the left flank region of NSG mice and were grouped randomly and treated with 2A2-OSU-2S-ILP (n=7), IgG-OSU-2S-ILP (n=6) or 2A2-Empty-ILP (n=6). Harvested tumor masses reveal diminished tumor growth in 2A2-OSU-2S-ILP treated mice ($P < 0.0001$ Vs 2A2-Empty-ILP).

Supplemental Figure 1:

(A) Activity of OSU-2S in MCL cell lines REC1 and Z-138 by MTS assay. Cells ($1 \times 10^6/\text{mL}$) were incubated with varying concentrations of OSU-2S and mitochondrial activity was measured at 24 and 48hrs after treatment. Bars represent mean with SE.

(B) Histogram overlay showing ROR1 expression in normal B and MCL B cells. Normal B cells isolated from healthy donors (N=3) and MCL patients (N=3) derived B cells were stained with polyclonal anti-human ROR1 and assessed by flow cytometer for ROR1 expression. ROR1 fluorescent intensity by median(X-Med), mean(X-AMean) and geometric mean(X-GMean) were displayed.

(C) Inhibition of lymphoma growth by ROR1 targeted OSU-2S nanoparticles. Proliferation of Mino cells engrafted into the left flank region of NSG mice was measured using digital calipers. Mice were treated with different treatments as indicated. Tumor volume in cm^3 was calculated using the formula $(\text{length} \times \text{width}^2)/2$. Bars represent mean $\pm$ SE.

(D) CD74 induction in MCL primary cells and in JeKo and Mino cell lines treated with OSU-2S for 24hours. MCL primary cells ($2 \times 10^6/\text{ml}$), JeKo and Mino cells ($1 \times 10^6/\text{ml}$) were cultured with OSU-2S ($4 \mu\text{M}$ primary cells and $5 \mu\text{M}$ for JeKo and Mino) or FTY720 ($12.5 \mu\text{M}$ JeKo and $7.5 \mu\text{M}$ Mino) for 24hours and were analyzed by flow cytometer for CD74 expression.

Figures:

Figure 1

Figure 2

Supplemental Figure 1

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