Supplemental Methods

Cell lines: NIH-H929 (H929) and RPMI-8226 myeloma cell lines were obtained from ATCC. Dr. Steven Rosen (Northwestern University) kindly provided MM1.S and MM1.R cells. OP2M, U266 and KMS-11 cells were obtained through Dr. Arul Chininnian (University of Michigan). Mantle cell lymphoma Z138 and Mino cells were kindly provided by Dr. Richard Ford (M.D. Anderson Cancer Center). Non-Hodgkin’s lymphoma cell lines SUDHL-4, SUDHL-10, OCI LY-1, OCI LY-3, OCI LY-10 and L428 Hodgkin’s lymphoma cells were kindly provided by Dr. Ryan Wilcox (University of Michigan). Cell were grown and maintained in RPMI-1640 with 10% heat-inactivated FBS (Atlanta Biologicals) and 2 mM L-glutamine. HCT116 and DLD-1 Usp9x+/+ and Usp9x-/- cells were provided by Dr. Bunz (Johns Hopkins University) and maintained in McCoy’s 5A medium with 6% heat-inactivated FBS (Atlanta Biologicals) and 2 mM L-glutamine.

Quantitative PCR for Usp9x and Usp24: Bone marrow aspirates obtained from myeloma patients who signed a University of Michigan IRB-approved consent were used to enrich myeloma plasma cells. Plasma cells were isolated from myeloma bone marrow by the EasySep (Stem Cell Technologies) method. RNA from 500,000 plasma cells was prepared by QIAGEN Rneasy micro kit. Whole transcriptome from the RNA was amplified using QuantiTect kit (Qiagen). For the quantitation of Usp9X and Usp24 real-time quantitative PCR primers were obtained from Realtimeprimers.com. Quantitation was performed by the SYBR green method using a BioRad CFX96 detection system.

Tissue microarray analysis of Usp9x and Usp24: Formalin-fixed, paraffin-embedded tissue blocks (FFPE) of mantle cell lymphoma (MCL) or chronic lymphocytic leukemia (CLL) cases were obtained from the files of the Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI. The University of Michigan Institutional Review Board provided a waiver of informed consent to obtain these samples. After pathological review, a tissue microarray was constructed from the most representative area using the methodology of Nocito et al 17. Each case was represented by two 1 mm diameter cores, obtained from the most representative, non-necrotic area of the tumor. Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO LSAB+ and diaminobenzadine (DAB) as the chromogen. De-paraffinized TMA sections were labeled with Usp9x (rabbit polyclonal antibody, 1:1000, AbCam, Cambridge Ma, Ab26334) or Usp24 (rabbit polyclonal antibody, 1:50, AbCam, Ab111088) for 60 min at ambient temperature. Microwave citric acid epitope retrieval was used prior to staining. Appropriate negative (no primary antibody) and positive controls were stained in parallel with each set of tumors studied. Antigen expression was scored semi-quantitatively as 0, 1+, 2+, or 3+ based on the intensity of cytoplasmic staining of the cells (0 = no
staining; 1+ = low intensity and/or patchy staining; 2+ = moderate intensity; 3+ = widespread, intense staining).

siRNA-mediated gene knockdown: RPMI-8226 cells at a density of 5 x 10^4/ml were transfected with 50 nM siRNA (SMARTpool siRNA, ThermoScientific) using 5 μl/ml RNAiMax (Invitrogen) following the protocol provided by Invitrogen. The transfected cells were incubated for 72 h and then harvested for western blot and apoptotic assessment. The SmartPool siRNA’s utilized included the following products: Usp9X: L-006099-00-0005, Usp24: L-006073-00-0005, Mcl-1: L-004501-00-0005 and scrambled: D-001810-10-20.

shRNA-mediated Usp9X and Usp24 knockdown: HEK293T cells were transfected with the retroviral packaging vectors pCL10A1 together with the LMP shRNA retroviral vectors to produce virus using PolyFect as described by the manufacturer (QIAGEN). The medium was changed to DMEM with 10% fetal bovine serum and after 48 h, the viral supernatant was collected and 2 μl Lipofectamine (Invitrogen) was added per ml of virus supernatant. The cells were spin infected at 2300 g for 2 hours at 32°C and fresh media was added. Two days after infection, the medium was changed and 1 μg/ml of puromycin was added. After puromycin selection (7 days), viable cells were recovered by ficoll gradient and Usp9x and Usp24 levels were examined by immunoblotting or cell survival was analyzed by Annexin V-Alexa 647/propidium iodide staining using a BD Caliber flow cytometer.

Xenograft studies – NSG [NOD/SCID/IL2r-g (null)] mice were subcutaneously injected into the mid dorsal region with 5 x 10^6 MM1.S cells suspended in an equal volume of media and Matrigel. Tumors were allowed to establish to about 100 mm^3, after which mice were tumor size matched and allocated into each treatment group (vehicle, or G9). The first day of treatment was day 28 post-inoculation. Treatment was by daily ip injection with G9 (15, 10, 5, 2.5 mg/kg) in DMSO:PEG:PBS (4:5:1). Tumor size was determined by caliper measurements [width(2) x length x height/2] every 2 days. All animal work was conducted under a University of Michigan Animal Care and Use (UCUCA) approved protocol and conforms to all relevant regulatory standards.

Plasmids - LMP-HS1 (Usp9x-1), LMP-HS2 (Usp9x-2), LMP-24-1 (Usp24-1), LMP-24-2 (Usp924-2), Mcl1-1, Mcl1-2 and control LMP-Luc were acquired from Openbiosystems. MIG-Flag-Usp9x - p3xFlag-Usp9x was created by 3-way cloning using PCR to amplify a 320 bp N-terminal fragment of Usp9x with the Stul site in Usp9x. Forward: 5’-tgttaggaagtctacagcactgtgtgctc-3’ Reverse: 5’-ggaaccacccatgagcc-3’. The PCR product was cut with HindIII and Stul. pCDNA5-TAP-Usp9x was cut with Stul and NotI. These fragments were ligated into p3XFlag-CMV10 (Sigma) linearized with HindIII/NotI. A PCR was performed
with forward primer 5’-gctctagatctatggactacaaagacc-3’ and the same reverse primer described above for p3XFlag-Usp9x. This product was cut with BglII and Stul, and ligated together with the Stul/BamHI fragment from p3XFlag-Usp9x together with MIGR1 linearized with BglII. The Mcl-1-V5 expression vector was received from Dr. Ulrich Maurer. pRK5-HA-Ub was obtained from Dr. Vaibhav Kapuria (University of Lausanne). HA-ΔN-Usp24 was subcloned from pENT223.1-USP24 (Genecopoeia; prod.id. HOC20345) after moving the PspOMI fragment into pcDNA3.1 PspOMI. Then pCDNA3.1-Usp24 was cut with Stul and cloned into pCMV-HA-N (Clontech) digested with MscI. All construct sequences were verified by PCR and Sanger sequencing.

For co-IP analysis 293T cells were transfected with the described expression vectors at equal amount of DNA totaling 6 ug DNA. Twenty-four hours later, cells were treated with 2 µM MG132 overnight and lysates prepared in 40 mM HEPES pH 7.4, 2 mM EDTA, 10 mM Na-pyrophosphate-50 mM NaF and protease inhibitors (Roche) with 0.5 mM DTT. The samples were sonicated and cleared by centrifugation at 16,000 g for 10 minutes at 4 °C. IP was done overnight with 250 ug protein and anti-HA antibody. The following day 30 ul of 50:50 protein A/G beads (GE) was added for 1 hour. The beads were washed with the buffer described above with 150 mM NaCl added followed by another 2 washes with buffer with no added salt. Beads were boiled in 20 ul of Laemli buffer. Endogenous co-IP in HCT116 and H929 cells was done on lysates of cells treated for 4 hours with 5 µM MG132 and lyzed in the buffer described above. IP was done with 500 ug of protein in the presence of 2 µM MG132 and 0.5 mM NEM for 4 hours with 1 ug of antibody and captured for one hour with 30 µl protein A/G, washed and boiled in 20 µl of Laemli buffer.

For analysis of deubiquitination activity of Usp24 on Mcl-1, Usp24 KD and control KD HEK293T cells were transfected with 5 µg of Mcl-1-V5 and 2 µg pRK5-HA-Ub. Lysates were prepared 36 hours later in RIPA buffer and 500 µg of pre-cleared lysate was immunoprecipitated with anti-V5 for 2 hours followed by 1 hour with 30 µl protein A/G at 4 °C. Beads were washed 3 times with buffer and boiled in 20 µl Laemli buffer. Western blot analysis was performed with the anti-HA antibody to detect ubiquitinated Mcl-1-V5.

Additional antibodies used in this study were purchased from the following sources: anti-actin (Sigma-Aldrich); anti-ubiquitin clone P4D1, goat, anti-rabbit/mouse/rat IgG-conjugated horseradish peroxidase, Mcl-1 (Santa Cruz Biotechnology); anti-Usp9x, Usp24 (Bethyl Laboratories); anti–poly(ADP-ribose) polymerase (PARP), (Cell Signaling Technology); anti-HA (clone 3F10; Roche Applied Science).

Cell proliferation and viability were measured as previously described.
Annexin V-Allophycocyanin(APC)/Propidium iodide staining was used to measure apoptosis in control and treated cells. Briefly, $10^5$ cells/ml were harvested from tissue culture plates and centrifuged at 2,000 g for 5 min at room temperature. Medium supernatant was removed and cells were washed once in PBS. Cells were then re-suspended in 0.4 ml of cold propidium iodide. Cells were then re-suspended in 0.4 ml of annexin binding buffer with 1 µg/ml propidium-iodide with annexin-V-APC (Biolegend). Samples were incubated on ice for 5 minutes in the dark, and analyzed on the FACScalibur (Becton Dickinson). An Annexin V-fluorescein isothiocyanate (FITC) staining assay was also performed to measure apoptosis in myeloma and CD34+ cells as previously described.¹⁷

Statistical analysis – Data points are shown as the mean ± S.D. Student’s t test was used to assess statistical performance using GraphPad Prism 6 and GraphPad InStat3.

**Legends for Supplemental Figures**

**Supplemental Figure S1.** shRNA-mediated knockdown of Usp9x and Usp24, primary myeloma DUB activity and deubiquitination of Mcl-1 by Usp24.

A. Control (LMP) and Usp9x-specific (HS1, HS2) shRNA viral constructs were used to infect MM1.S cells. After puromycin selection (3 days), cell were expanded for 4 days and lysates were screened for Usp9x expression by immunoblotting. Actin was blotted as a protein loading control. Control and Usp24-specific (USP24-1, USP24-2) shRNA viral constructs were used to infect H929 cells. After 3 days of puromycin selection and cell expansion for 4 days, Usp24 protein levels were analyzed by immunoblotting. HSP90 was blotted as a protein loading control.

B. Cells harvested one or 2 days after selection were screened for changes in survival by measuring Annexin V staining in duplicate samples. Each bar represents the average +/- S.D. of duplicate assays. ** indicate p> 0.05 calculated using the Student’s t test.

C. Increased expression of Usp9x and Usp24 as compared to normal bone marrow. Direct comparisons of normal bone marrow to either MGUS or myeloma, and MGUS to myeloma. Oncomine™ was used to perform this analysis. Fold changes and p values provided based on Oncomine algorithms.

D. DUB activity was measured by labeling protein extracted from $10^6$ CD138+ plasma cells, CD138+ myeloma cells or lineage depleted plasma cell leukemia cells (equal cell number input). DUB assay was performed and resolved on a 4-15% gradient gel and immunoblotted with an anti-HA antibody. Usp9x and Usp24 levels were sequentially analyzed after stripping of the blot.

E. Deubiquitination of Mcl-1 by Usp24. To assess the ability of Usp24 to deubiquitinate Mcl-1, HEK293T cells expressing Usp24 shRNA and control shRNA were transfected with V5 tagged Mcl-1 and HA-
Ubiquitin. Lysates were immunoprecipitated with the anti-V5 antibody and immunoblotted for the HA-tagged ubiquitin. IP’ed McI-V5 and Usp24 protein levels are shown. Hsp70 is shown as a loading control for the HEK293T shRNA control and Usp24 KD cells.

**Supplemental Figure S2. Usp9x and Usp24 expression in normal tissues and B-cell malignancies.**

A. Tissue microarrays containing normal tissue sections (as noted – top) and specimens derived from 14 mantle cell lymphoma patients were stained for Usp9x (left) or Usp24 (right) and visualized as described in the Methods. Triplicate sections for each tumor block are shown. Six tumors (MCL1, MCL5, MCL6, MCL8, MCL12 and MCL13) showed evidence of increased Usp9x expression compared to normal tissue. Usp24 was elevated in liver, but was minimal detected in other tissues. Elevated Usp24 was detected in MCL1, MCL2, MCL7, MCL8, MCL9, MCL11 and MCL12. Elevated expression of both Usp9x and Usp24 was detected in MCL1, MCL8 and MCL9.

B. Tissue microarrays containing normal tissue sections (as noted – top) and specimens derived from 14 chronic lymphocytic leukemia patients were stained for Usp9x (left) or Usp24 (right) and visualized as described in the Methods. Triplicate sections for each tumor block are shown. Four tumors (CLL2, CLL3, CLL4 and CLL9) showed evidence of increased Usp9x expression compared to normal tissue. Elevated Usp24 was detected in CLL3, CLL4 and CLL8. Elevated expression of both Usp9x and Usp24 was detected in CLL3 and CLL4.

**Supplemental Figure S3. Activity of a novel DUB inhibitor.**

A. Concentration–dependent Usp9x catalytic domain (Usp9xCD) activity inhibition by WP1130 (left) and G9 (right). Usp9xCD was prepared and purified as previously described. Activity assessment was performed in duplicate using Ub-AMC as DUB substrate. Samples were pre-incubated with the indicated concentration of inhibitor for 30 min at 37°C before measurement of enzymatic activity as previously described. Enzymatic activity was assessed by monitoring changes in fluorescence over time (shown) IC50 values for each compound (Table 2) were calculated using GraphPad6 Prism software.

B. Concentration-dependent cytotoxicity of WP1130 and G9. MM1.S and Z138 cells were plated at 10^5 cells per well of a 96 well plate and incubated with the indicated concentration of compound for 48 hours before assessing growth and/or survival by MTT staining as previously described. Each data point represents the average +/- S.D. of triplicate assays.

C. Concentration-dependent cell survival in DUB inhibitor treated myeloma and normal CD34+ cells. Myeloma cells (H929, MM1.S; red bars) and normal CD34+ cells derived from two independent
transplant donors (Miltenyi bead CD34+ enriched; blue bars) were treated with the indicated concentration of G9 for 24 hours before assessing cell viability by Annexin V staining. Each bar represents the average +/- S.D. of two myeloma cell lines or patient samples (CD34+) conducted in triplicate in two independent experiments.

D. Specific inhibition of Usp24 by G9. HCT116 WT (Usp9x+/+) and Usp9x−/− cells were treated for 6 hours with 5 µM G9. The lysates were used for DUB activity assessment with vinyl-sulfone-HA-Ubiquitin and resolved on a 4-15% gradient gel and immunoblotted with anti-HA. Hsp70 is loading control for the DUB assay. Forty microgram of the same lysates was assessed for the expression of Usp9x and Usp24. Actin was probed as a protein loading control.

E. Concentration-dependent DUB inhibition and Mcl-1 regulation by WP1130 and G9. Z138 cells were treated with the indicated concentration of compound for 4 hours before assessing DUB activity changes as reported in figure 1. Usp9x, Usp24 and Mcl-1 levels in equal protein cell lysates were also examined by immunoblotting. Actin was probed as a protein loading control.

Supplemental Figure S4. Cytotoxicity of G9 in myeloma and diffuse large B-cell lymphoma (DLBCL).

Myeloma (top) cell lines with known chromosomal abnormalities or DLBCL (bottom) cell lines with subclass distinctions were incubated with G9 for 72 hours before assessing cell growth/viability changes by MTT assay. IC50 values were calculated with GraphPad Prism 6 software. Each value represents the average +/- S.D. of two independent assays of triplicate samples.

Supplemental Figure S5. In vivo anti-myeloma activity of a novel DUB inhibitor.

A. Tumor bearing mice treated for 2 weeks with G9 at the dose indicated (figure 7A) were left untreated and tumor growth was monitored until tumors approached maximally allowable tumor volumes. The results represent the average tumor volume +/- S.D. from 5 mice per group.

B. NSG mice were inoculated with MM1.S tumor cells as described in the Methods. Mice in groups of 5 were divided into equal initial tumor burden (day1) and treated at the G9 dose indicated daily for 1 week. Tumor volumes were measured at day 3 and 7 and each value represents the average tumor volume in 5 mice +/- S.D. p-values are enclosed in the figure.
Supplemental Table 1. Clinical and cytogenetic characteristics of patients whose tumor samples were evaluated in this study

<table>
<thead>
<tr>
<th>MM Patient ID</th>
<th>Time since diagnosis (y)</th>
<th>Treatment regimens</th>
<th>Response to last treatment</th>
<th>Poor cytogenetic prognoses</th>
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</thead>
<tbody>
<tr>
<td>6821</td>
<td>6</td>
<td>Bortezomib-doxorubicin-dexamethasone, tositumomab, thalidomide maintenance, ASCT</td>
<td>Very good partial response followed by relapse</td>
<td>Absent</td>
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<tr>
<td>7261</td>
<td>4</td>
<td>Bortezomib-dexamethasone-thalidomide-cisplatin-doxorubicin-cyclophosphamide-etoposide, tositumomab, tandem ASCT, bortezomib-dexamethasone-thalidomide, lenalidomide-bortezomib-dexamethasone, cytarabine-lenalidomide-bortezomib-dexamethasone</td>
<td>Progressive disease</td>
<td>ND</td>
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<tr>
<td>5500</td>
<td>New diagnosis of plasmacytoma and plasma cell leukemia</td>
<td>48 hrs of radiation</td>
<td>Naïve disease</td>
<td>17p and 13 del, t(14;16)</td>
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<tr>
<td>5281</td>
<td>New diagnosis of MM</td>
<td>None</td>
<td>Naïve disease</td>
<td>del 13 and t(4;14)</td>
</tr>
<tr>
<td>5609</td>
<td>7</td>
<td>Bortezomib-doxorubicin-dexamethasone, ASCT</td>
<td>Progressive disease</td>
<td>Trisomy 3 and 9, 13 del</td>
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<tr>
<td>4283</td>
<td>5</td>
<td>Lenalidomide-bortezomib-doxorubicin-dexamethasone, cyclophosphamide-bortezomib-dexamethasone</td>
<td>Stable disease</td>
<td>Trisomy 3, 7, 9, 11, and 15, 14q32 del</td>
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<tr>
<td>0823</td>
<td>New diagnosis of plasma cell leukemia</td>
<td>None</td>
<td>Naïve disease</td>
<td>Normal</td>
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</table>

ASCT, autologous stem cell transplantation; ND, not determined.
Supplemental Table 2. Comparison of compound solubility, activity, microsomal metabolism, initial pharmacokinetics, and toxicity.

<table>
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<th>Parameter</th>
<th>WP1130</th>
<th>EOAi3402143</th>
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<tr>
<td><strong>Aqueous Solubility [μM]</strong></td>
<td>2.3</td>
<td>80 (HCl salt)</td>
</tr>
<tr>
<td><strong>Enzyme IC50 (Usp9x Cat. Dom.) [μM]</strong></td>
<td>4.8</td>
<td>1.6</td>
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<tr>
<td><strong>Liver Microsomes t1/2 [min]</strong></td>
<td>&gt;60</td>
<td>40</td>
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<tr>
<td><strong>Mouse PK</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV (20 mg/kg) t1/2 [min]</td>
<td>90</td>
<td>45</td>
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<tr>
<td>ng/ml (0.5 hours)</td>
<td>531</td>
<td>1980</td>
</tr>
<tr>
<td>ng/ml (4 hours)</td>
<td>56</td>
<td>193</td>
</tr>
<tr>
<td>IP (20 mg/kg) t1/2 [min]</td>
<td>90</td>
<td>30-45</td>
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<tr>
<td>ng/ml (0.5 hours)</td>
<td>215</td>
<td>1920</td>
</tr>
<tr>
<td>ng/ml (4 hours)</td>
<td>119</td>
<td>120</td>
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<tr>
<td><strong>Toxicity (LD50)</strong></td>
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<tr>
<td>IV [mg/kg]</td>
<td>&lt;30</td>
<td>30</td>
</tr>
<tr>
<td>IP [mg/kg]</td>
<td>40</td>
<td>&gt;50</td>
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IC50, drug concentration causing 50% inhibition of the desired activity; LD50, lethal dose (individual dose required to kill 50% of a population); t1/2, half-life (the amount of time required for a quantity to fall to half its value as measured at the beginning of the time period).
Figure S1.

A. 

- **LMP**
- **HS1**
- **HS2**

  - **Usp9x**
  - **Actin**

  **MM1S Cells**

- **Control**
- **USP24-2**
- **USP24-1**

  - **Usp24**
  - **HSP90**

  **H929 Cells**

B. 

- **% Survival**

  - **Day1**
  - **Day2**

  - **LMP**
  - **HS1**
  - **HS2**

  **MM1S cells**

C. 

- **USP9X**
  - **Fold change: 1.380**
  - **p = 0.008**

  **MGUS**
  **Myeloma**

- **USP24**
  - **Fold change: 1.482**
  - **p = 9.99E-4**

  **MGUS**
  **Myeloma**

D. 

- **Plasma Cells**
- **Myeloma plasma cells**
- **Plasma cell leukemia**

  - **250kDa**
  - **Usp9x/Usp24 DUB Activity**
  - **250kDa**
  - **Usp9x**
  - **250kDa**
  - **Usp24**

E. 

- **Sh-Luciferase**
- **sh-USP24-2**

  - **IP: anti-V5**
  - **WB: α-HA**
  - **IPed Mcl-1-V5**

  - **250kDa**
  - **150kDa**
  - **100kDa**
  - **75kDa**
  - **50kDa**
  - **37.5kDa**
  - **37kDa**

  - **Usp24**
  - **250kDa**
  - **75kDa**
  - **HSP70**

**293T**
Figure S3.

A. 

B. 

C. 

D. 

E.
Table 1: Summary of chromosomal abnormalities and IC50 values for myeloma cell lines and lymphoma cell lines.

<table>
<thead>
<tr>
<th>Myeloma Cell Lines</th>
<th>NCI-H929</th>
<th>MM1.S</th>
<th>RMPI-8226</th>
<th>OPM2</th>
<th>KMS-11</th>
<th>U266</th>
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<tr>
<td>Chromosomal Abnormalities</td>
<td>del13, hypodiploid t(4;14)</td>
<td>t(14;16)</td>
<td>del13, hypodiploid t(4;14)</td>
<td>t(4;14)</td>
<td>t(4;14)</td>
<td>del13, hypodiploid</td>
</tr>
<tr>
<td>IC50 nM</td>
<td>445 ± 98</td>
<td>515 ± 45</td>
<td>1922 ± 185</td>
<td>549 ± 15</td>
<td>655 ± 152</td>
<td>682 ± 55</td>
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<table>
<thead>
<tr>
<th>Lymphoma Classification</th>
<th>Diffuse Large B Cell Lymphoma (DLBCL)</th>
<th>Classical Hodgkin’s Lymphoma (cHL)</th>
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<tr>
<td>Cell Line</td>
<td>Myc and Bcl-2 rearrangement with IgH locus</td>
<td>Germinal Center B-cell like Lymphoma (GCB)</td>
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<tr>
<td>SUDHL-10</td>
<td>SUDHL-4</td>
<td>OCI LY-1</td>
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<tr>
<td>IC50 nM</td>
<td>680 ± 10</td>
<td>1309 ± 13</td>
</tr>
</tbody>
</table>
Figure S5.

A. Tumor Volume (cm³) vs Treatment Interval

- Control
- 2.5 mg/kg

B. Tumor Volume (mm³) vs Days of Treatment

- Control
- 10 mg/kg
- 15 mg/kg

Legend:
- * p<0.05
- ** p<0.01
- *** p<0.001

vs Control

Time after initial G9 Treatment