Methods

Cell lines and Cell Culture

The Nalm-6 (ACC-128) and RCH-ACV (ACC-548) cell lines were purchased from DSMZ. REH (CRL-8286) and SUP-B15 (CRL-1929) cell lines were purchased from ATCC. Primary patient samples were de-identified and obtained through the Duke University Department of Medicine, Division of Hematologic Malignancies and Cellular Therapy biorepository under an IRB-approved protocol (#00082876). STR profiling was repeated routinely to authenticate cell lines. Mycoplasma testing by PCR was performed every three months on all cell lines in culture and all testing was negative for the duration of this study. Nalm-6, REH and RCH-ACV cells were cultured in RPMI 1640 (Corning Inc.) supplemented with 10% FBS (Gemini Bio-Products). Sup-B15 cells were cultured in Iscove's modified Dulbecco's medium (IMDM), supplemented with 20% FBS and 0.05 mM 2-mercaptoethanol. Primary patient cells were cultured in StemSpan SFEM II (STEMCELL Technologies). All cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere. For all *in vitro* assays, unless stated otherwise, copanlisib (MedChemExpress), GS-649443 (Gilead Sciences, Inc.) and Ara-C (Hospira Inc.) were used at a concentration of 100 nM.

Western Blot Analysis

At sample collection 1×10^6 cells were centrifuged and washed with ice-cold PBS. Ice-cold SDS loading buffer (Cell Signaling Technology) containing fresh reducing agent (Cell Signaling Technology), protease inhibitor cocktail (ThermoFisher) and phosphatase inhibitor cocktail (Cell Signaling Technology) was added at 200 µl to the cell pellet. The suspension was transferred into a centrifuge tube and placed on ice for 10 min. The centrifuge tube was placed in a sonicator bath (Symphony™, model: 97043-936; VWR) for 5 minutes followed by a 5-minute incubation at 95°C. The suspension was centrifuged at 14,000 x*g* for 15 min at 4 °C and supernatants (total cell lysate) were used immediately or stored at −80 °C. Samples were analyzed using the following primary antibodies, as indicated: anti-phosphorylated-Akt (Ser473) (Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-β-actin (Abcam) anti-phosphorylated GSK-3β (Ser9) (Cell Signaling Technology), anti-GSK-3β (Cell Signaling Technology), GAPDH (Cell Signaling Technology), anti-PI3K p110α (Cell Signaling Technology), anti-PI3K p110β (Cell Signaling Technology), anti-PI3K p110δ (Cell Signaling Technology) and anti-PI3K p110γ (Cell Signaling Technology). Horseradish peroxidase (HRP)-coupled anti-rabbit IgG (Cell Signaling Technology) and goat anti-mouse IgG–HRP

(ThermoFisher) were used as secondary antibodies, and immunoreactive proteins were detected by enhanced chemiluminescence (ECL) (ThermoFisher). Densitometry was quantified using ImageJ software.

Flow Cytometry Analysis

Cells were treated with vehicle or copanlisib for 72 hours in RPMI + 0.3% FBS before CD49f (integrin α6) staining. For *in vitro* cell surface receptor staining, 1×10^6 cells per 100 µl were stained with antibody for 30 min at 4 °C in Automacs buffer (BD Biosciences) containing 3% BSA, washed with 3% BSA, and analyzed on a FACS Canto II cytometer. For apoptosis analysis, cells were resuspended in Annexin V Binding Buffer (BD Biosciences) and stained with either anti-annexin V–FITC or anti-annexin V-PE antibody and propidium iodide or 7-AAD for 15 min at room temperature and analyzed. For cell cycle analysis, cells were permeabilized in 70% ethanol overnight at −20 °C, washed and resuspended in Automacs buffer containing 3% BSA and then incubated with anti-human-KI-67 for 1 h at 4 °C before washing with 3% BSA and staining with 4 µg ml^{-1} Hoechst 34580 (Invitrogen) at 1×10^6 cells per 100 µl Automacs buffer containing 3% FBS for 30 min at room temperature. The following antibodies were purchased from BD Pharmingen: peridium chlorophyll protein complex with cyanin-5.5 (PerCP–Cy5.5)-conjugated mouse antibody against human CD10, APC-conjugated rat antibody against mouse CD45, PE-conjugated rat antibody against human CD49f, Alexa Fluor647-conjugated annexin-V antibody, PE-conjugated KI-67 antibody, PE-conjugated mouse antibody against human CD19 and AF647-conjugated antibody against active caspase 3.

Adhesion Assay

Recombinant human laminin 511 (Biolamina) was plated overnight on 96-well tissue culture plates (10 µg/mL). Residual laminin was rinsed off with warm media and then 25×10^3 leukemic cells were plated in 100 µL of media (± 0.05 mM $MnCl_2$). The cells were allowed to adhere for 20-30 minutes before treating with vehicle or copanlisib (100 nM) for 45 minutes. After treatment, the wells were washed 3x with warm PBS and then fixed and stained with 5 µg ml^{-1} Hoechst 33258 (Molecular Probes–Life Technologies). Plates were imaged on a Leica SP8 confocal microscope with a 10x objective lens (Leica Camera AG, Germany) with LAS X software. Adhesion indices were calculated as the number of cells attached after washing divided by the number of cells attached in a “no wash” control.

Three-Dimensional Invasion Assay

Cells were treated with vehicle or copanlisib (100 nM) for 72 h in 0.3% serum. Cells were resuspended in serum-free rat-tail collagen (Advanced Biomatrix 5153 at 3 mg ml^{-1}) alone or supplemented with laminin (Sigma-Aldrich, L2020) to a final concentration of 0.01 mg ml^{-1}. Resuspended cells were aliquoted into 96-well plates and spun down to the bottom of the plate. Collagen was allowed to polymerize for 2 h and cell culture medium (with 100 ng/ml copanlisib) was added on top of the gel as a chemoattractant. After 16 h of incubation at 37 °C, plates were fixed and stained with 5 µg ml^{-1} Hoechst 33258 (Molecular Probes–Life Technologies). Plates were imaged on a Leica SP8 confocal microscope with a 10x objective lens (Leica Camera AG, Germany) with LAS X software. The three-dimensional migration index was calculated as number of invading cells at 50 µm divided by the total number of cells.

Immunofluorescence

Laminin-coated plates were made as described above. Leukemic cells (25 x 10^3) were plated in 100 µL of media and allowed to adhere for 30 minutes before treating with vehicle or copanlisib (100 nM) for another 45 minutes. Cells were fixed with 4% PFA for 10 minutes, permeabilized with 0.3% Triton and blocked in 2% BSA in PBS. The cells were stained with phosphorylated Myosin Light Chain 2 antibody (CST, 3671S) for 2 hours at RT followed by a 2 hour secondary staining with anti-rabbit IgG HRP-linked antibody at RT. Plates were imaged on a Leica SP8 confocal microscope with a 25x water objective lens (Leica Camera AG, Germany) with LAS X software.

Proliferation Assays

For single agent studies cells were treated with copanlisib or GS-649443 for 72 h. For chemotherapeutic/PI3Ki combination studies, cells were treated with 100 nM Ara-C for 3 hours followed by 3x PBS washes and subsequent treatment with copanlisib or GS-649443 (100 nM). For corticosteroid/PI3Ki combination studies, cells were treated with copanlisib or GS-649443 (100 nM) with or without dexamethasone (Nalm-6, 100 nM; RCH-ACV, 500 nM; Sup-B15, 5 nM). Cells were counted using a hemocytometer and trypan blue (Gibco) was used to discriminate between live and dead cells.

Collection of Bone Marrow and CSF Cells

Mouse femurs were collected and BM cells were aspirated with RPMI1640 containing 10% FBS. The spine was removed from mice and carefully separated into individual vertebral bodies by cutting through the intervertebral discs. The spinal cord and meninges of each vertebral body

were then washed with RPMI1640 containing 10% FBS to collect cells from the CSF. The BM and CSF cells were passed through 70 μm filters, washed with PBS and treated with ACK lysis buffer to remove red blood cells.

Mouse Engraftment

Specific pathogen-free 6- to 8-week old male and female SCID mice (breeders purchased from Charles River Labs) were inoculated intravenously with 5×10^6 Nalm-6-GFP or RCH-ACV cells in phosphate-buffered saline through the tail vein. All experimental procedures involving mice were approved by the Animal Care and Use Committee of Duke University. All experiments were performed in accordance with the relevant ethical guidelines and regulations.

Drug Administration

Copanlisib was formulated in PEG400/H_20 (20/80, v/v) acidified water solution (2% trifluoroacetic acid) and brought to approximately pH 5.5 for *in vivo* administration. For single agent studies copanlisib was administrated to Nalm-6-GFP or RCH-ACV cell-engrafted mice by tail vein injection from day 1 after engraftment. Mice were dosed twice a day, twice weekly with 14 mg/kg copanlisib. For chemotherapeutic/PI3Ki combination studies, Ara-C (Hospira Inc.) was administered by intraperitoneal injection at 500 mg/m^2 every day for 5 days from day 20 after engraftment with Nalm-6 GFP cells. Copanlisib (14 mg/kg) or vehicle was administered twice a day on days 21 and 25. GS-649443 (2 mg/kg) or vehicle was administered twice daily for 7 days from day 20 by oral gavage. For Nalm-6 β-catenin reporter GFP engrafted mice, Ara-C was administered as above on day 20 only and GS-649443 was administered from day 20 twice a day for 3 days by oral gavage. For paired-animal analysis, mice from the vehicle and PI3Ki treated groups were paired at the beginning of the study. Both mice in each pair were sacrificed when either of them showed clinical endpoint symptoms. For all studies mice were monitored daily and sacrificed when they showed hind limb paralysis or other significant CNS symptoms, severe cachexia (≥20% weight loss), respiratory or other distress, or extreme lethargy.

Histology

Tissues were dehydrated with 70% ethanol once for 25 minutes, 95% ethanol twice for 30 minutes, and 100% ethanol twice for 45 minutes. Tissues were then soaked in xylene three times for 30 minutes and soaked in paraffin (Polyfin #19280-01) twice for 45 minutes at 60 °C. Tissues were then mounted into block with paraffin. Tissues were sectioned into 5 μm sections, floated in a 42 °C water bath, and mounted onto adhesive slides (Leica Biosystems #3800080).

Slides were air-dried overnight. Prior to staining, slides were baked in an oven at 37 °C overnight. Slides were deparaffinized by two changes of xylene for five minutes, two changes of 100% ethanol for five minutes, one change of 95% ethanol for five minutes, and one change of 70% ethanol for five minutes. Slides were rinsed with tap water and then stained with Gill 3x Hematoxylin (Fisherbrand #245-657) for 30 seconds. Slides were then rinsed with tap water and tissues were differentiated with 1% acid alcohol (1% HCl in 70% ethanol) and slides were rinsed with tap water. Tissues were then soaked in bluing reagent (Fisherbrand #245-681) for 30 seconds and slides were rinsed with tap water. Slides were then soaked in ethanol briefly and tissues were stained with eosin (Leica #3801601). Slides were then dehydrated once in 95% ethanol for one minute and twice in 100% ethanol for one minute. Sides were soaked in twice in xylene for one minute, and cover slips (Thermo Scientific #3323) were mounted onto the slides with mounting medium (Tissue-Tek # 6419).

Statistical Analysis

Kaplan–Meier curves with two-sided log rank Mantel–Cox analysis were used to assess *in vivo* survival. Two-sided paired Student's *t*-tests were used for analysis of vehicle versus inhibitor effects on tumour burden in mice. One-way ANOVA analyses with Tukey post-hoc testing for multiple comparisons were performed for B-ALL integrin α6 expression comparisons, B-ALL proliferation assays, β-catenin reporter activity following Ara-C/copanlisib treatment and apoptosis assays (Annexin V). Two-sided paired Student's *t*-tests were used for adhesion assays, invasion assays, immunofluorescence quantification, G0 arrest comparisons, caspase 3 apoptosis assay and β-catenin reporter/pAkt activity and proliferation following Ara-C/GS-649443 treatment. The majority of statistical analysis was performed using GraphPad Prism version 7.0, while JMP was used for some of the cell cycle analysis. Significant p values were defined as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Supplemental Figures

Supplemental Figure 1. PI3Kδi treatment does not cause apoptosis of B-ALL cells in the BM of leukemic mice.

A, Representative flow cytometry analysis of Annexin V/PI staining of blasts isolated from BM of Nalm-6-GFP engrafted mice treated with GS-649443. **B,** Quantification of A. Data are mean ± s.e.m.; unpaired two-sided Student's t test.

Supplemental Figure 2. Serum deprivation induces Akt phosphorylation in B-ALL cells *in vitro.*

A, Representative flow cytometry analysis of phosphorylated Akt at Thr308 in Nalm-6 cells grown in complete media or deprived of FBS for 24 hours. **B,** Quantification of C. Data are mean ± s.e.m.; unpaired two-sided Student's t test, $n = 3$ biologically independent experiments; *p<0.05.

Supplemental Figure 3. Cytarabine induces Akt and GSK3β phosphorylation in B-ALL cells *in vitro*.

Western blot analysis of Akt and GSK3β phosphorylation in Nalm-6 and RCH-ACV cell lines at multiple time points following 3 hour exposure to Ara-C. Data shown are independent biological replicates of the data presented in Figure 1A.

Supplemental Figure 4. PI3Kδi treatment decreases cytarabine-induced β-catenin signaling activity and Akt phosphorylation

Dual pAkt and β-catenin reporter GFP positivity detected over time by flow cytometry in Nalm-6 cells treated with vehicle or GS-649443 after 3 hour Ara-C exposure. Data are mean ± s.e.m.; unpaired two-sided Student's *t*-test, $n = 3$ biologically independent experiments; *p<0.05, **p<0.01

Supplemental Figure 5. GS-649443 has a modest effect on the proportion of BM blasts in G0/1 and S phase.

Cell cycle analysis of Nalm-6 isolated from the BM of mice treated with vehicle or GS-649443 from day 1 post-engraftment until clinical endpoint (treatment schema, Fig. 2A). Data are mean ± s.e.m.; unpaired two-sided Student's t test

Supplemental Figure 6. Copanlisib inhibits B-ALL cell proliferation more potently than GS-649443.

A – C, B-ALL cell proliferation over 72 hours of treatment with vehicle or 100 nM, 1 μM or 10 μM GS-649443 or copanlisib. Data are mean ± s.e.m.; $n = 3$ technical replicates.

Supplemental Figure 7. Copanlisib causes an arrest in the G0/1 phase of the cell cycle in B-ALL cell lines.

A-D, Flow cytometry analysis using Hoechst staining indicates a cell cycle arrest in B-ALL (Nalm-6 – REH) cell lines at G0/G1 following treatment over time with copanlisib. Data are mean ± s.e.m.; ANOVA, *n* = 3 biologically independent experiments; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplemental Figure 8. Copanlisib treatment does not trigger apoptosis of B-ALL cell lines *in vitro*.

A-D, Cell death over time in B-ALL cell lines treated with copanlisib vs. vehicle determined by Annexin V and PI staining quantified by flow cytometry. Data are mean ± s.e.m.; unpaired two-sided Student's *t*-test, *n* = 3 biologically independent experiments; *p<0.05.

Supplemental Figure 9. Copanlisib triggers apoptosis of primary patient B-ALL cells *in vitro*.

Apoptosis in primary patient B-ALL cells as detected by Annexin V + Annexin V/PI or Annexin V/7-AAD. Cells were treated with vehicle, 100 nM GS-649443, or 100 nM copanlisib for 72 hours.

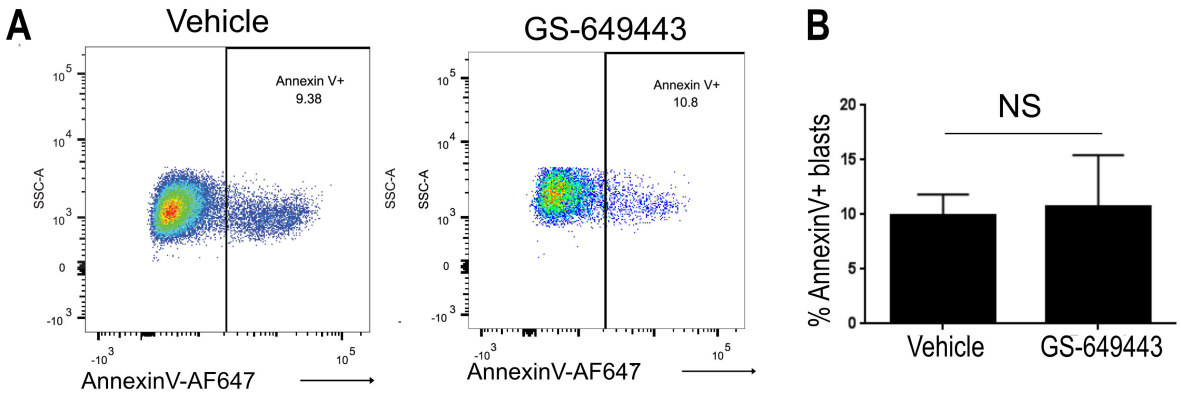
Supplemental Figure 10. Pan-PI3K inhibition with copanlisib more potently decreases BM and CNS disease burden than δ isoform-selective PI3K inhibition with GS-649443.

A – D, Fold change in B-ALL disease burden in BM and CSF of leukemic mice treated with vehicle, GS-649443, or copanlisib. **A,C**, are data originally reported in Yao et al. Nature 2018 [8] and presented alongside new data in **B,D** to summarize the differential effects of copanlisib vs. GS-649443 on BM and CNS disease.

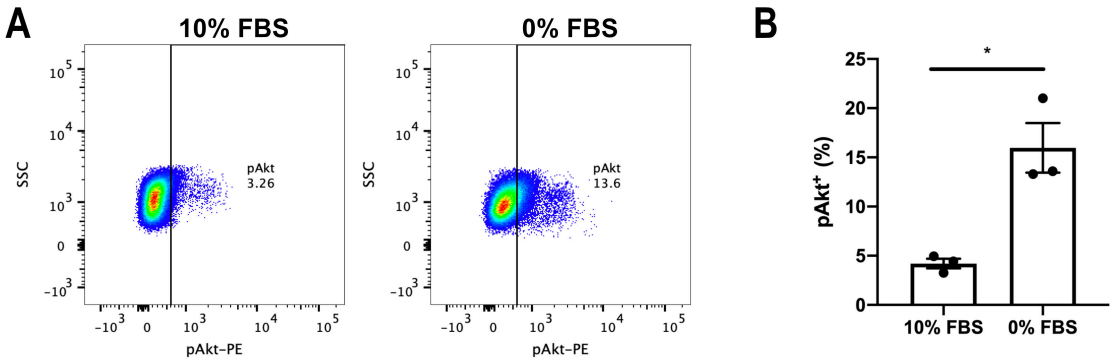
Supplemental Table 1. Cell line information

Cell line	Subtype	Clinical details	Cell source	Fusion gene	Cytogenetics*
Nalm-6	B-cell ALL	19 year old with CNS relapse	Peripheral blood	N/A	46(43-47)<2n>XY, t(5;12)(q33.2;p13.2)
RCH-ACV	B-cell ALL	8 year old with BM relapse	BM	TCF3-PBX1	hyperdiploid with 2% polyploidy - 43-50<2n>XX, +8, t(1;19)(q23;p13.3)
SUP-B15	B-cell ALL	8 year old with 2nd BM relapse	BM	BCR-ABL	46(44-47)<2n>X, -X, +16, del(3)(p22), t(4;12;21;16)(q32;p13;q22;q24.3)-inv(12)(p13q22), t(5;12)(q31-q32;p12), der(16)t(16;21)(q24.3;q22)
REH	B-cell ALL	15 year old with BM relapse and hyperleukocytosis	Peripheral blood	ETV6-RUNX1	46<2n>XY, der(1)t(1;1)(p11;q31), add(3)(q2?7), der(4)t(1;4)(p11;q35), t(9;22)(q34;q11), add(10)(q25), ?del(14)(q23q31), der(16)t(9;16)(q11;p13)

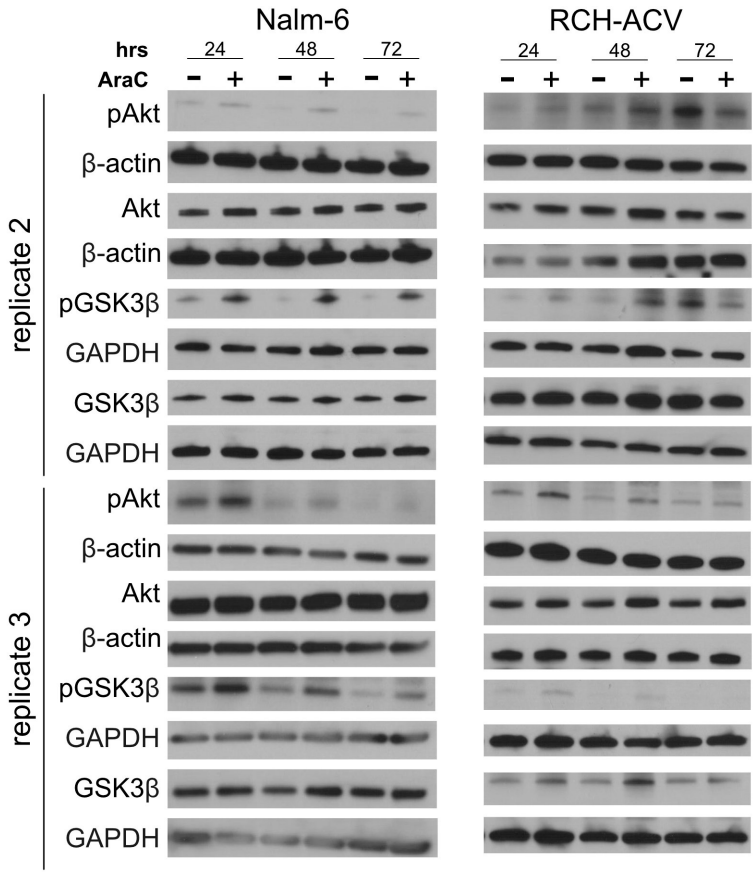
*cytogenetics from DSMZ



Supplemental Figure 1



Supplemental Figure 2



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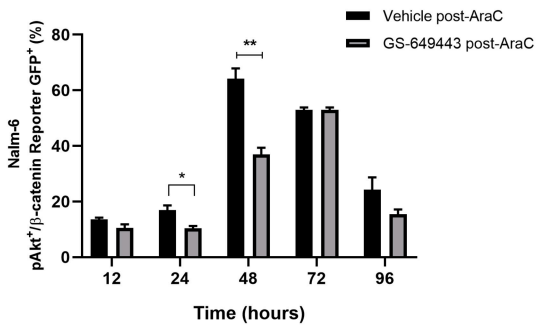

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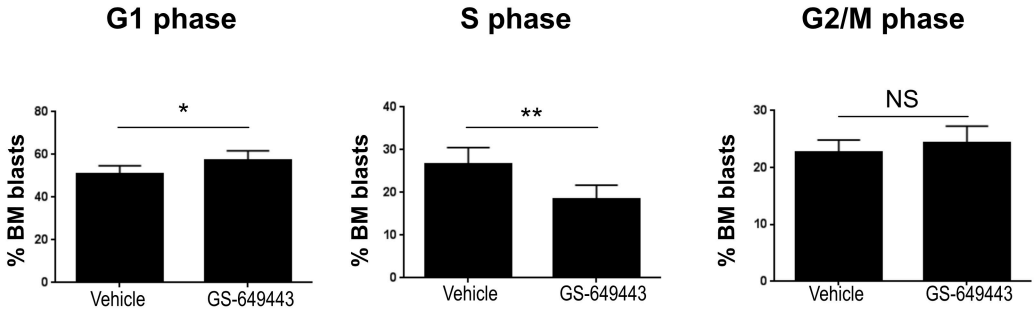

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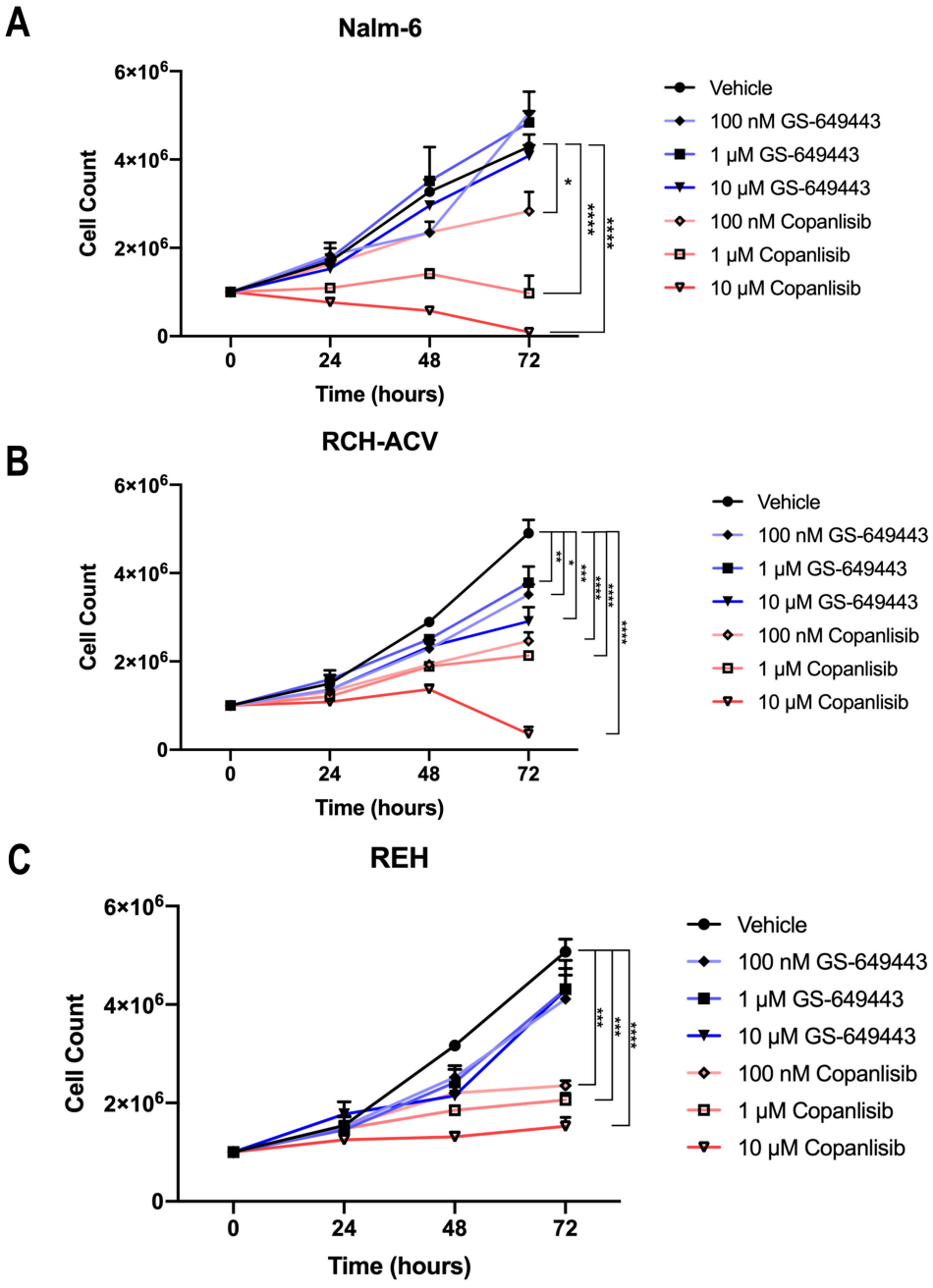

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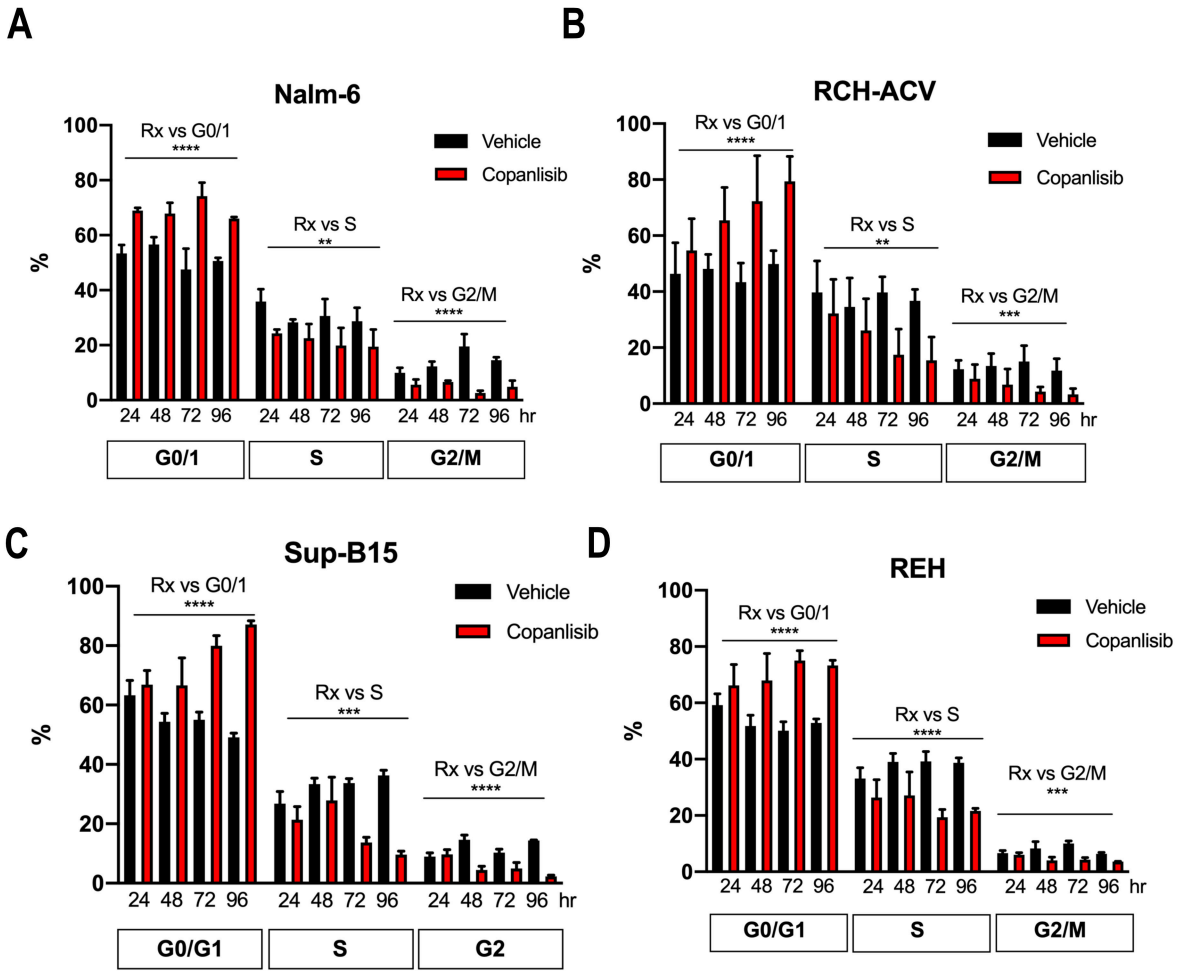

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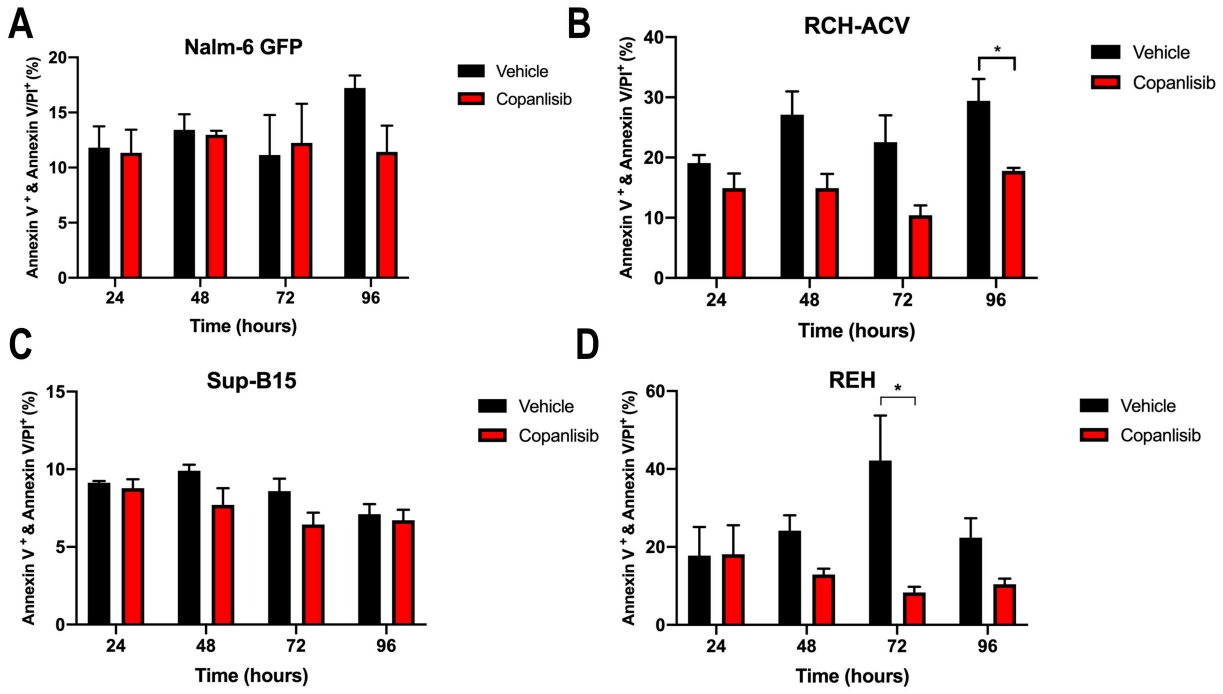

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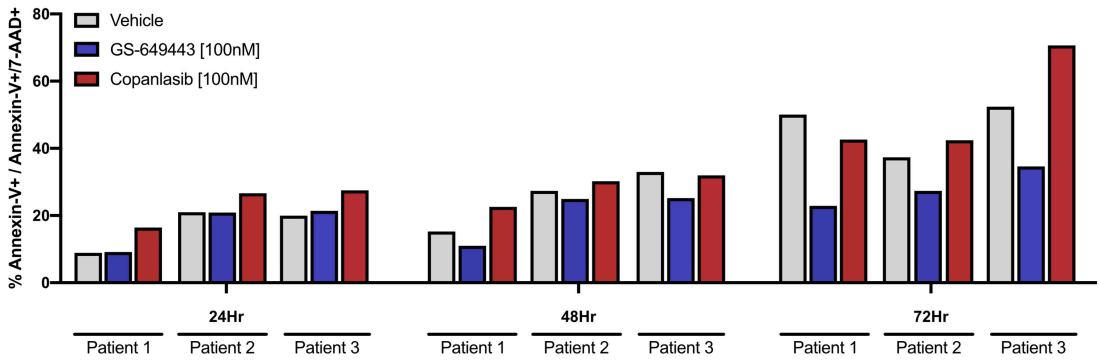
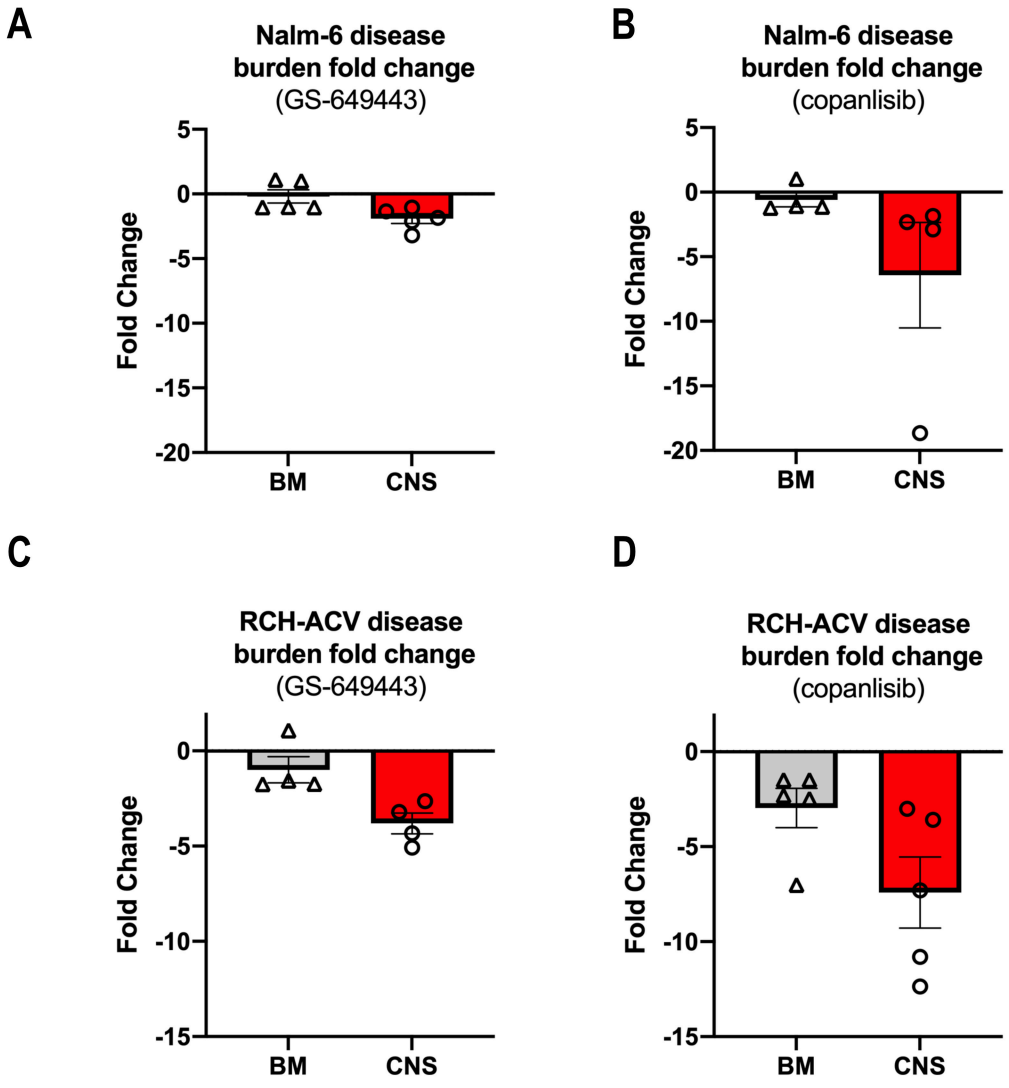


Supplemental Figure 9



Supplemental Figure 10