Supplementary Information

SHMT inhibition is effective and synergizes with methotrexate in T-cell acute lymphoblastic leukemia

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Supplementary Materials and Methods

Proliferation assays

Standard proliferation assays were conducted in 96 well plates and relative cell number measured using resazurin sodium salt. For adherent cell lines, $3x10^3$ cells were plated in each well in 100 μL of media were allowed to adhere overnight. Media containing inhibitor (0.5% DMSO final concentration) was added the next day and cell growth was assessed via fluorescence intensity using a Synergy HT plate reader (BioTek Instruments) at 48 hours post-treatment. For suspension cell lines, 10^4 cells were plated in each well in 90 μL of media and allowed to rest for 2 h. 10 μL of a 10x solution of inhibitor in media (0.5% DMSO final concentration) were added and cell growth was assessed via fluorescence intensity using a Synergy HT plate reader (BioTek Instruments) at 48 hours post-treatment.

Cell cycle and apoptosis analyses

Cell cycle and apoptosis were analyzed in cells treated during 48 h with vehicle (DMSO) or (+) SHIN2 (IC_{50} respective to each cell line). PI/RNase Staining Buffer (BD Pharmingen) was used to analyze cell cycle distribution. Apoptosis was quantified with the PE-AnnexinV Apoptosis

Detection Kit I (BD Pharmingen). Stained cells were analyzed using an Attune NxT Flow Cytometer (Thermo Fisher Scientific) and data was analyzed using the FlowJo software (BD).

Flow cytometry analysis of T-cell development

Single cell suspensions of thymocytes were obtained by pressing the thymus through a 40 μm filter. For flow cytometry-based analysis of discrete stages of T-cell development, cells were stained with anti-mouse fluorochrome-conjugated antibodies as follows (clone names are provided in parentheses): CD8a phycoerythrin (PE) (1:200, 53-6.7, BD Pharmigen), CD4 APC (1:1000, RM4-5, BD Pharmigen), CD44 eFluor 450 (1:200, IM7, Thermo Fisher Scientific) and CD25 Alexa Fluor 488 (1:1000, 7D4, Thermo Fisher Scientific). Total thymus populations were represented in a CD4 versus CD8a plot and CD4/CD8 DP ($CD4^{+}CD8a^{+}$), CD4SP ($CD4^{+}CD8a^{-}$), CD8SP ($CD4^{-}CD8a^{+}$) and CD4/CD8 DN ($CD4^{-}CD8a^{-}$) populations were gated. Then CD4/CD8 DN were plotted in a CD44 versus CD25 plot to characterize DN1 ($CD4^{-}CD8a^{-}CD44^{+}CD25^{-}$), DN2 ($CD4^{-}CD8a^{-}CD44^{+}CD25^{+}$), DN3 ($CD4^{-}CD8a^{-}CD44^{-}CD25^{+}$) and DN4 ($CD4^{-}CD8a^{-}CD44^{-}CD25^{-}$) populations. Flow cytometry data were collected on an Attune NxT Flow Cytometer (Thermo Fisher Scientific) and analyzed with FlowJo software (BD).

Measurement of hematological parameters

Blood collected from mice treated with (+)SHIN2 and/or methotrexate was analyzed using an Element HT5 Veterinary Hematology Analyzer (Heska).

Cell metabolism studies

HCT116 cells were plated in 6 cm tissue culture dishes and grown to 60% confluency. At the start of an experiment, the appropriate media was added to cells, which included vehicle (DMSO 0.5%), or (+) or (-) SHIN2 2 μM and/or sodium formate 1 mM. Total incubation time was 24 h

and fresh media was added 6 h before metabolome extraction for untargeted metabolomics. Cell number was estimated by the measurement of packed cell volume (PCV).

U-^{13}C-serine was purchased from Cambridge Isotope Laboratories. Serine isotopically labeled media was prepared from glucose, glycine and serine-free RPMI media (R9660, Teknova) and supplemented with 10% dialyzed FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Human T-ALL cell lines (i.e. Molt3, Molt4 and Jurkat) were plated in 6 cm tissue culture dishes at 10^6 cells/mL in the appropriate media, which included vehicle (DMSO 0.5%), or (+)SHIN2 2 μM and/or sodium formate 1 mM. Total incubation time was 24 h. 6 h before metabolome extraction, cell density was adjusted to 2×10^6 cells/mL in fresh media. In the case of incubations with U-^{13}C-serine, serine isotopically labeled media was used for the 6 h incubation. Cell number was counted directly using Trypan blue and the Countess system (Invitrogen).

Metabolite extraction

For HC116 cells, media was removed by aspiration and the cells washed once with room temperature PBS. Metabolome extraction was performed by the addition of 800 μL of ice cold extraction solvent (40:40:20 acetonitrile:methanol:water + 0.5% formic acid). After a 1 min incubation on ice, the extract was neutralized by the addition of NH_4HCO_3. The samples were incubated at -20 °C for ~30 min, at which point the wells were scraped and the extract transferred to Eppendorf tubes and centrifuged (15 min, 16000 rpm, 4 °C). The resulting supernatant was frozen on dry ice and kept at -80 °C until LC-MS analysis.

For T-ALL cells, cell suspensions were transferred to 1.5 mL tubes and pelleted (30 s, 6000 rpm, room temperature). Media was removed by aspiration and the pellet washed once with room temperature PBS. Metabolome extraction was performed by the addition of 75 μL of ice cold solvent (40:40:20 ACN:MeOH:H2O + 0.5% formic acid) to the pellet. After a 5 min incubation

on ice, acid was neutralized by the addition of NH_4HCO_3. After centrifugation (15 min, 16000 rpm, 4 °C), the resulting supernatant was transferred to a clean tube, frozen on dry ice and kept at -80 °C until LC-MS analysis.

For serum, 5 µL were mixed with 150 µL -20 °C 80:20 methanol:water, vortexed, and immediately centrifuged at 16,000 x g for 10 min at 4 °C. The supernatant was collected for LC-MS analysis.

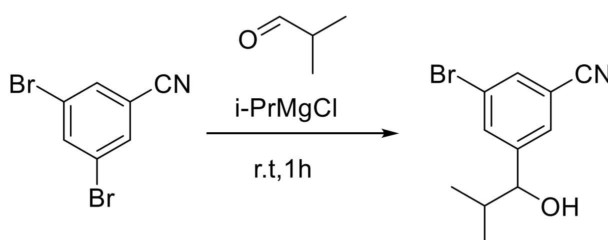
LC-MS-based untargeted metabolomics

Extracts were analyzed using a quadrupole–orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific) coupled to hydrophilic interaction chromatography via electrospray ionization. Liquid chromatography separation was on a XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 µm particle size; Waters) using a gradient of solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 water:acetonitrile, pH 9.45) and solvent B (acetonitrile). Flow rate was 150 µl/min, column temperature was 25 °C, autosampler temperature was 5 °C and injection volume was 10 µl. The liquid chromatography gradient was: 0 min, 90% B; 2 min, 85% B; 3 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 14 min, 25% B; 16 min, 0% B; 21 min, 0% B; 22 min, 90% B; 25 min, 90% B. Autosampler temperature was 5 °C and injection volume was 10 µl. The mass spectrometer was operated in negative-ion mode to scan from m/z 70 to 1,000 at 1 Hz and a resolving power of 140,000. Data were analyzed using the El-MAVEN software (v 0.2.4, Elucidata), with compounds identified based on exact mass and retention time match to commercial standards. Isotopic labeling of metabolites arising from incubation with U-^{13}C-serine were corrected for natural abundance, as previously described (ref. 52). Adherent cell metabolite abundances were normalized by packed cell volume; suspension cells to cell count.

Chemical synthesis of SHIN2

Commercially available starting materials were used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on Varian or Bruker 500, 400, or 300 MHz spectrometers and referenced to solvent peaks. Coupling constants (J) are reported in hertz, chemical shifts are reported in δ (ppm) as either s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), dd (doublet of doublets), or m (multiplet).

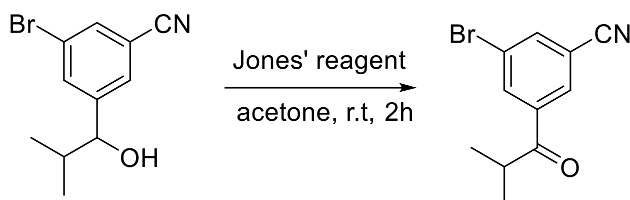
Synthesis of 3-bromo-5-(1-hydroxy-2-methylpropyl) benzonitrile:



Into a 5000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed 3,5-dibromobenzonitrile (300 g, 1150 mmol, 1 equiv), THF (2500 mL). This was followed by the addition of i-PrMgCl (579 mL, 5630 mmol, 1 equiv, 2M) dropwise with stirring at 4 °C. The resulting solution was stirred for 1 h at room temperature. To this was added a solution of 2-methylpropanal (91.2 g, 1260 mmol, 1.1 equiv) in THF (700 mL) dropwise with stirring at room temperature. The resulting solution was stirred for 1 h at room temperature. One more reaction was repeated in parallel, combined and worked up together. The reaction was then quenched by the addition of 1000 mL of NH_4Cl (aq). The resulting solution was extracted with 3x2000 mL of ethyl acetate and the organic layers combined and dried over anhydrous sodium sulfate and concentrated. The residue was applied onto a silica gel column with ethyl acetate/petroleum ether (1:10). This resulted in 510 g (87.27%) of 3-bromo-5-(1-hydroxy-2-methylpropyl) benzonitrile as yellow oil. H-NMR- 3-bromo-5-(1-hydroxy-2-methylpropyl)

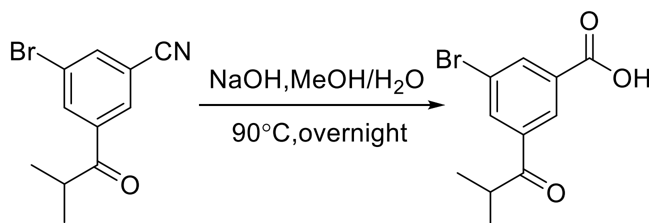
benzonitrile: ^{1}H NMR (300 MHz, Chloroform-d) δ 7.72 (d, J = 11.0 Hz, 1H), 7.62 – 7.51 (m, 1H), 4.48 (d, J = 5.9 Hz, 1H), 0.92 (dd, J = 14.8, 6.8 Hz, 6H).

<u>Synthesis of 3-bromo-5-(2-methylpropanoyl) benzonitrile:</u>



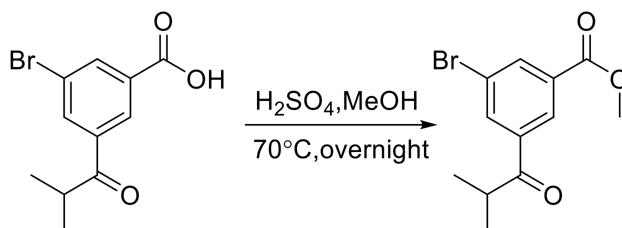
Into a 3000 mL 3-necked round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed 3-bromo-5-(1-hydroxy-2-methylpropyl)benzonitrile (255 g, 1000 mmol, 1 equiv) and acetone (2500 mL). This was followed by the addition of Jones reagent (294 mL) dropwise with stirring at room temperature. The resulting solution was stirred for 2 h at room temperature. This reaction was repeated one time and worked up together. The reaction was then quenched by the addition of 1000 mL of water. The resulting solution was extracted with 3x1000 mL of ethyl acetate and the organic layers combined and dried over anhydrous sodium sulfate and concentrated. The residue was applied onto a silica gel column with ethyl acetate/petroleum ether (1:10). This resulted in 430 g (84.98%) of 3-bromo-5-(2-methylpropanoyl) benzonitrile as a white solid. H-NMR-3-bromo-5-(2-methylpropanoyl) benzonitrile: ^{1}H NMR (300 MHz, Chloroform-d) δ 8.29 (t, J = 1.7 Hz, 1H), 8.15 (t, J = 1.5 Hz, 1H), 7.97 (t, J = 1.7 Hz, 1H), 3.52 – 3.43 (m, 1H), 1.26 (d, J = 6.9 Hz, 6H).

<u>Synthesis of 3-bromo-5-(2-methylpropanoyl) benzoic acid:</u>



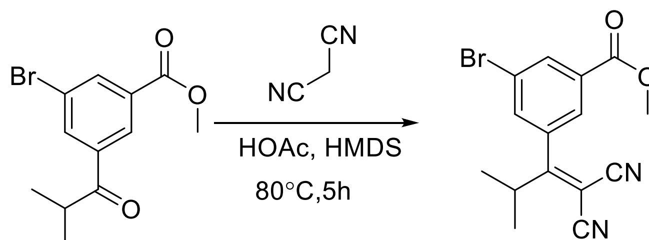
Into a 2000 mL round-bottom flask, was placed 1-bromo-3-isocyano-5-(2-methylpropanoyl) benzene (143 g, 567 mmol, 1 equiv) in MeOH (500 mL), NaOH (541 mL, 2160 mmol, 4 M). The resulting solution was stirred for overnight at 90 °C. The reaction repeated two times. The resulting mixture was concentrated. The pH value of the solution was adjusted to 6 with HCl (12 M). The solids were collected by filtration. The solid was dried under infrared light. This resulted in 460 g (99.71%) of 3-bromo-5-(2-methylpropanoyl) benzoic acid as a white solid. H-NMR-3-bromo-5-(2-methylpropanoyl) benzoic: ^{1}H NMR (300 MHz, DMSO-d6) δ 8.39 (t, J = 1.5 Hz, 1H), 8.23 (t, J = 1.8 Hz, 1H), 8.16 (t, J = 1.8 Hz, 1H), 3.72 – 3.63 (m, 1H), 1.11 (d, J = 6.6 Hz, 6H).

Synthesis of methyl 3-bromo-5-(2-methylpropanoyl) benzoate:



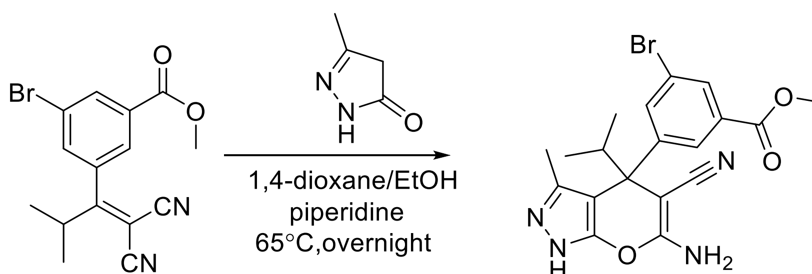
Into a 10 L 3-necked round-bottom flask, was placed 3-bromo-5-(2-methylpropanoyl) benzoic acid (460 g, 1700 mmol, 1 equiv) in MeOH (4500 mL), H_2SO_4 (500 mL, 0.510 mmol) was added dropwise with stirring at room temperature. The resulting solution was stirred overnight at 80 °C.The resulting mixture was concentrated. The reaction was poured into 200 g of crushed ice. The solids were collected by filtration. The solid was dried under infrared light. This resulted in 460 g (95.08%) of methyl 3-bromo-5-(2-methylpropanoyl) benzoate as a white solid. H-NMR-3-bromo-5-(2-methylpropanoyl) benzoate: ^{1}H NMR (300 MHz, DMSO-d6) δ8.40 – 8.36 (m, 2H), 8.28 (t, J = 1.7 Hz, 1H), 3.91 (s, 3H), 3.82 – 3.63 (m, 1H), 1.11 (d, J = 6.6 Hz, 6H).

Synthesis of methyl 3-bromo-5-(1, 1-diisocyano-3-methylbut-1-en-2-yl) benzoate:



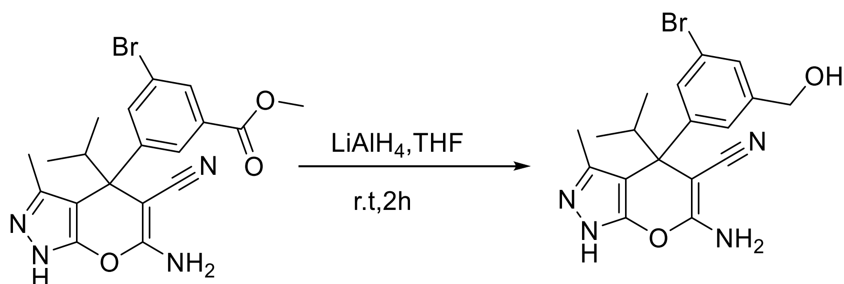
Into a 2000 mL 3-necked round-bottom flask, was placed methyl 3-bromo-5-(2-methylpropanoyl) benzoate (115 g, 403 mmol, 1 equiv) in HOAc (500 mL), diisocyanomethane (133 g, 2020 mmol, 5 equiv) and HMDS (197 g, 1220 mmol, 3 equiv) was added. The resulting solution was stirred for 5 h at 80 °C. This reaction is repeated in parallel for three times and worked up together. The reaction mixture was poured into 600 g of water/ice. The solids were collected by filtration. The solid was dried under infrared light. This resulted in 410 g (76.28%) of methyl 3-bromo-5-(1, 1-diisocyano-3-methylbut-1-en-2-yl) benzoate as an orange solid. H-NMR-methyl 3-bromo-5-(1, 1-diisocyano-3-methylbut-1-en-2-yl) benzoate: ^{1}H NMR (300 MHz, DMSO-d6) δ 8.21 (t, J = 1.5 Hz, 1H), 7.94 (dt, J = 7.1, 1.6 Hz, 2H), 3.91 (s, 3H), 3.35 – 3.26 (m, 2H), 1.10 (d, J = 6.6 Hz, 6H).

Synthesis of methyl 3-[6-amino-5-cyano-4-isopropyl-3-methyl-1H-pyrano[2,3-c]pyrazol-4-yl]-5-bromobenzoate:



Into a 1000 mL 3-necked round-bottom flask, was placed methyl 3-bromo-5-(1,1-dicyano-3-methylbut-1-en-2-yl) benzoate (50 g, 150 mmol, 1 equiv) in dioxane (200 mL) and EtOH (200 mL), piperidine (14 g, 0.16 mmol) and 3-methyl-5-pyrazolone (15 g, 0.15 mmol) was added. The resulting solution was stirred for overnight at 65 °C. The reaction was repeated seven times. The resulting mixture was concentrated. The residue was applied onto a silica gel column with ethyl acetate/petroleum ether (1:2). The crude product was purified by recrystalization with ethyl acetate. This resulted in 220 g (42.49%) of methyl 3-[6-amino-5-cyano-4-isopropyl-3-methyl-1H-pyrano[2,3-c]pyrazol-4-yl]-5-bromobenzoate as a white solid. H-NMR-KDG-0112-6: ^{1}H NMR (300 MHz, DMSO-d6) δ 12.24 (s, 1H), 7.96 (t, J = 1.5 Hz, 1H), 7.87 (s, 1H), 7.74 (t, J = 1.8 Hz, 1H), 7.00 (s, 2H), 3.86 (s, 3H), 2.79 – 2.70 (m, 1H), 1.78 (s, 3H), 0.90 (d, J = 6.6 Hz, 3H), 0.78 (d, J = 6.6 Hz, 3H).

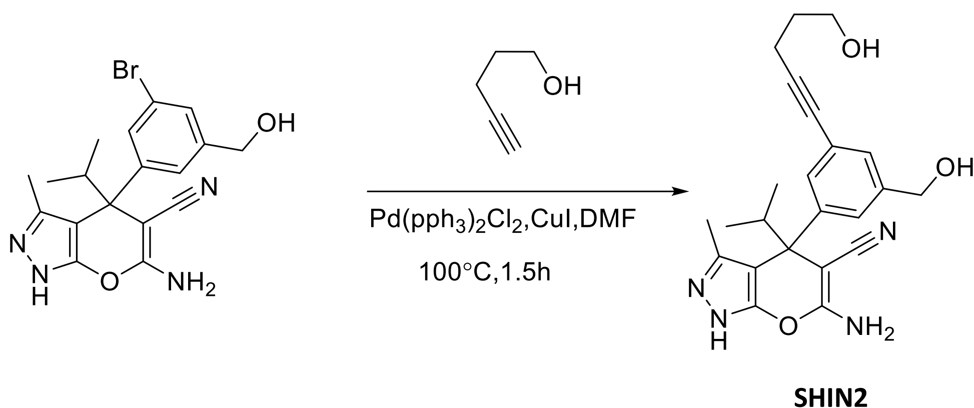
Synthesis of 6-amino-4-[3-bromo-5-(hydroxymethyl)phenyl]-4-isopropyl-3-methyl-1H-pyrano[2,3-c]pyrazole-5-carbonitrile:



Into a 1000 mL 3-necked round-bottom flask, was placed methyl 3-[6-amino-5-cyano-4-isopropyl-3-methyl-1H-pyrano [2, 3-c] pyrazol-4-yl]-5-bromobenzoate (50 g, 116 mmol, 1 equiv), THF (500 mL). This was followed by the addition of $LiAlH_4$ (8.8 g, 232 mmol, 2 equiv) in portions at room temperature. The resulting solution was stirred for 2 h at room temperature. Four reactions were repeated in parallel and worked up together. The reaction was then quenched by the addition of 10 mL of water. The pH value of the solution was adjusted to 6 with HCl (1 M). The resulting

solution was extracted with 3x1000 mL of ethyl acetate and the organic layers combined and dried over anhydrous sodium sulfate. The residue was applied onto a silica gel column with ethyl acetate. This resulted in 150 g (80.21%) of 6-amino-4-[3-bromo-5-(hydroxymethyl)phenyl]-4-isopropyl-3-methyl-1H-pyrano[2,3-c]pyrazole-5-carbonitrile as a white solid. H-NMR-6-amino-4-[3-bromo-5-(hydroxymethyl)phenyl]-4-isopropyl-3-methyl-1H-pyrano[2,3-c]pyrazole-5-carbonitrile: ^{1}H NMR (300 MHz, DMSO-d6) δ 12.18 (s, 1H), 7.36 (s, 1H), 7.29 (d, J = 1.8 Hz, 2H), 6.88 (s, 2H), 5.33 (t, J = 5.7 Hz, 1H), 4.48 (d, J = 5.7 Hz, 2H), 2.75 – 2.66 (m, 1H), 1.73 (s, 3H), 0.89 (d, J = 6.3 Hz, 3H), 0.76 (d, J = 6.3 Hz, 3H).

Synthesis of SHIN2 (6-amino-4-[3-(hydroxymethyl)-5-(5-hydroxypent-1-yn-1-yl)phenyl]-3-methyl-4-(propan-2-yl)-1H,4H-pyrano[2,3-c]pyrazole-5-carbonitrile):



Into a 40 mL round-bottom flask purged and maintained with an inert atmosphere of nitrogen, was placed 6-amino-4-[3-bromo-5-(hydroxymethyl)phenyl]-3-methyl-4-(propan-2-yl)-1H,4H-pyrano[2,3-c]pyrazole-5-carbonitrile (3 g, 7.44 mmol, 1 equiv), pent-4-yn-1-ol (1.25 g, 14.9 mmol, 2 equiv), DMF (15 mL, 0.205 mmol, 0.03 equiv), $Pd(PPh_3)_2Cl_2$ (0.52 g, 0.744 mmol, 0.1 equiv), CuI (0.28 g, 1.49 mmol, 0.2 equiv), Et_3N (2.26 g, 22.3 mmol, 3 equiv). The resulting solution was stirred for 1.5 h at 100 °C. And 35 reactions were repeated in parallel and worked up together. The resulting mixture was concentrated. The residue was applied onto a silica gel column with ethyl

acetate. The crude oil product (purity>90%) was slurried in ethyl acetate at room temperature for 4 h, and then filtered. This resulted (filter cake) in 45 g (42.52%) of SHIN2 as an off-white solid. H-NMR-SHIN2: ^{1}H NMR (300 MHz, DMSO-d6) δ 12.11 (s, 1H), 7.24 (s, 1H), 7.16 (d, J = 7.2 Hz, 2H), 6.83 (s, 2H), 5.23(t, J = 6 Hz, 3H), 4.54 – 4.45(m, 3H), 3.54 – 3.32 (m, 2H), 2.76 – 2.67 (m, 1H), 2.50 – 2.42 (m, 3H), 1.72 – 1.64 (m, 5H), 0.90 (d, J = 6.3 Hz, 3H), 0.77 (d, J = 6.3 Hz, 3H).

Chiral resolution: SHIN2 was separated by PREP-CHIRAL-HPLC with the following conditions: column: Lux Amylose-1 (2 x 250 mm); gradient: 20% ethanol/CO_2, 100 bar, 70 mL/min; detector, UV 220 nm.

Supplementary Figures

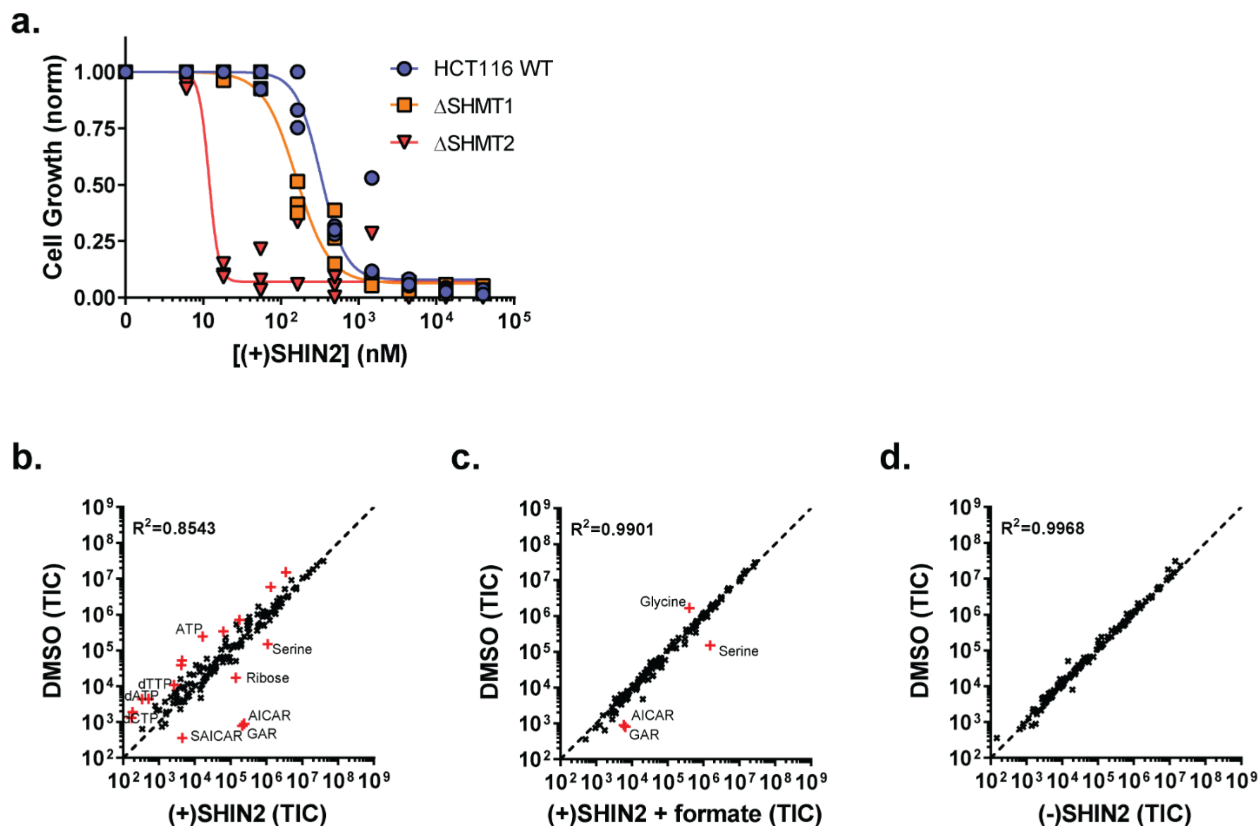


Figure S1, related to Fig.1 SHIN2 inhibits SHMT *in vitro*. (**a**) Growth of WT, ΔSHMT1 and ΔSHMT2 HCT116 cells incubated with increasing concentrations of (+)SHIN2 (n=3). (**b-d**) Metabolite levels in HCT116 cells (24 h drug exposure at 2 μM) (mean, n=3). Metabolites displaying a fold-change > 4 are highlighted in red.

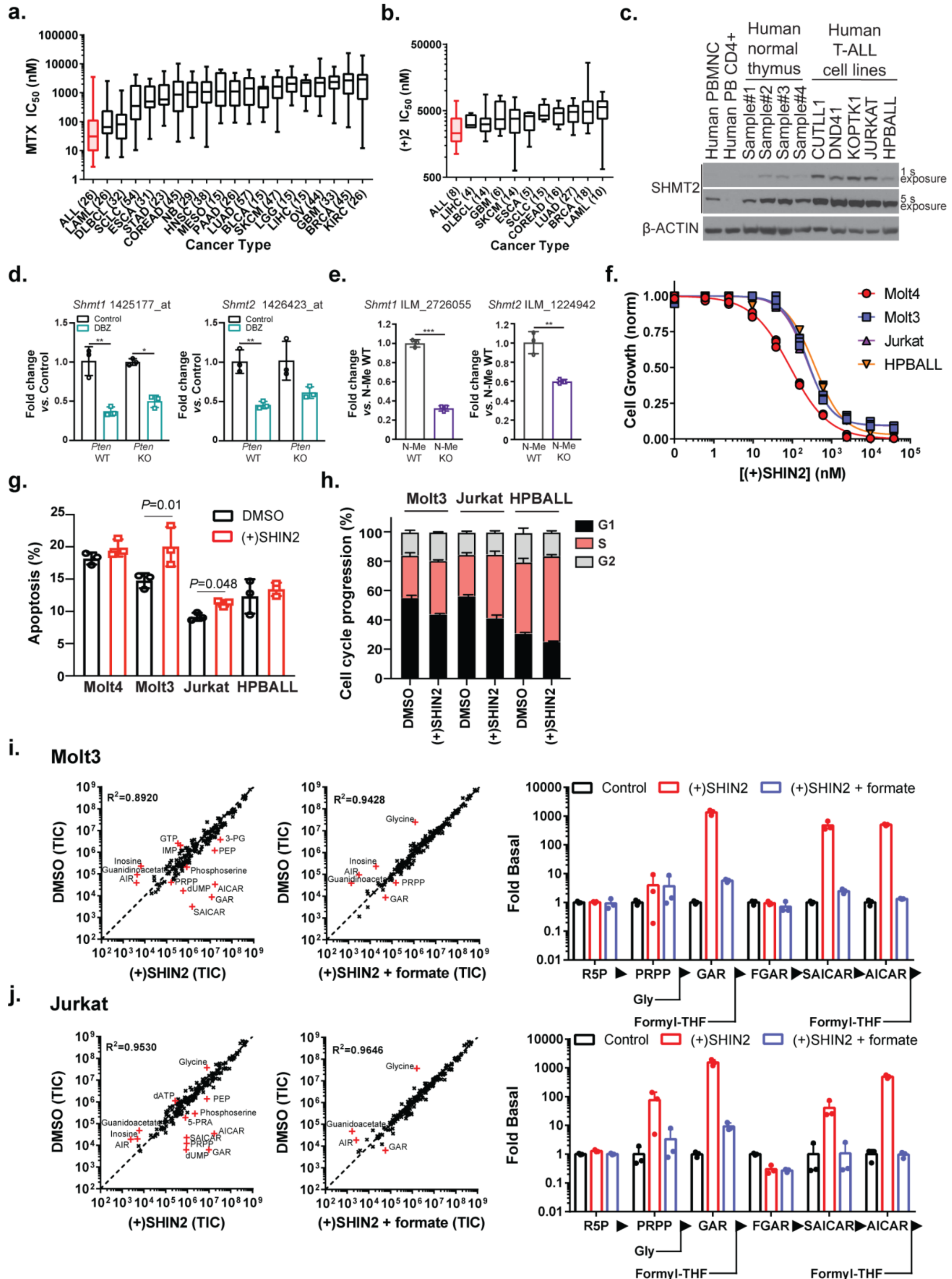


Figure S2, related to Fig.2. SHIN2 blocks growth of human T-ALL cell lines via SHMT inhibition. (**a**) IC_{50} values for methotrexate across several cell lines grouped by cancer type based on TGCA classification and ordered by median IC_{50} value (data obtained from www.cancerrxgene.org) (ref. 27). (**b**) IC_{50} values for the SHMT1/2 inhibitor (+)2 across several cell lines grouped by cancer type based on TGCA classification and order by median IC_{50} value (data obtained from ref. 18, data shown only for cells common to those included in panel A and with at least 5 cell lines per group. In **a** and **b**the number of cell lines per cancer type is indicated in parenthesis. (**c**) Western blots of SHMT2 (showing two different exposure time points) and β-ACTIN in normal hematological cells (i.e. human thymus, peripheral blood mononuclear cells and peripheral blood $CD4^+$ T cells) and various human T-ALL cell lines. (**d**) mRNA levels of *Shmt1* and *Shmt2* upon NOTCH1 inhibition with the DBZ gamma-secretase inhibitor in mouse T-ALL *in vivo* (taken from ref. 25) (FDR: *<0.05, **<0.005). (**e**) mRNA levels of *Shmt1* and *Shmt2* upon N-Me deletion in mouse T-ALL *in vivo* (taken from ref. 33) (FDR: **<0.005, ***<0.005). (**f**) Growth of the human T-ALL cell lines Molt4, Molt3, Jurkat and HPBALL (n=3). (**g**-**h**) Apoptosis (**g**, p value calculated using an unpaired two-tailed Student's t test) and cell cycle effects (**h**) in a panel of T-ALL cell lines treated with (+)SHIN2 for 48 h (mean ± SD, n=3) (**i-j**) Metabolite levels in Molt3 (**i**) and Jurkat cells (**j**) (24 h drug exposure) (mean, n=3). Metabolites displaying a fold-change > 4 are highlighted in red. (+)SHIN2 concentration is 2 μM, formate concentration is 1 mM.

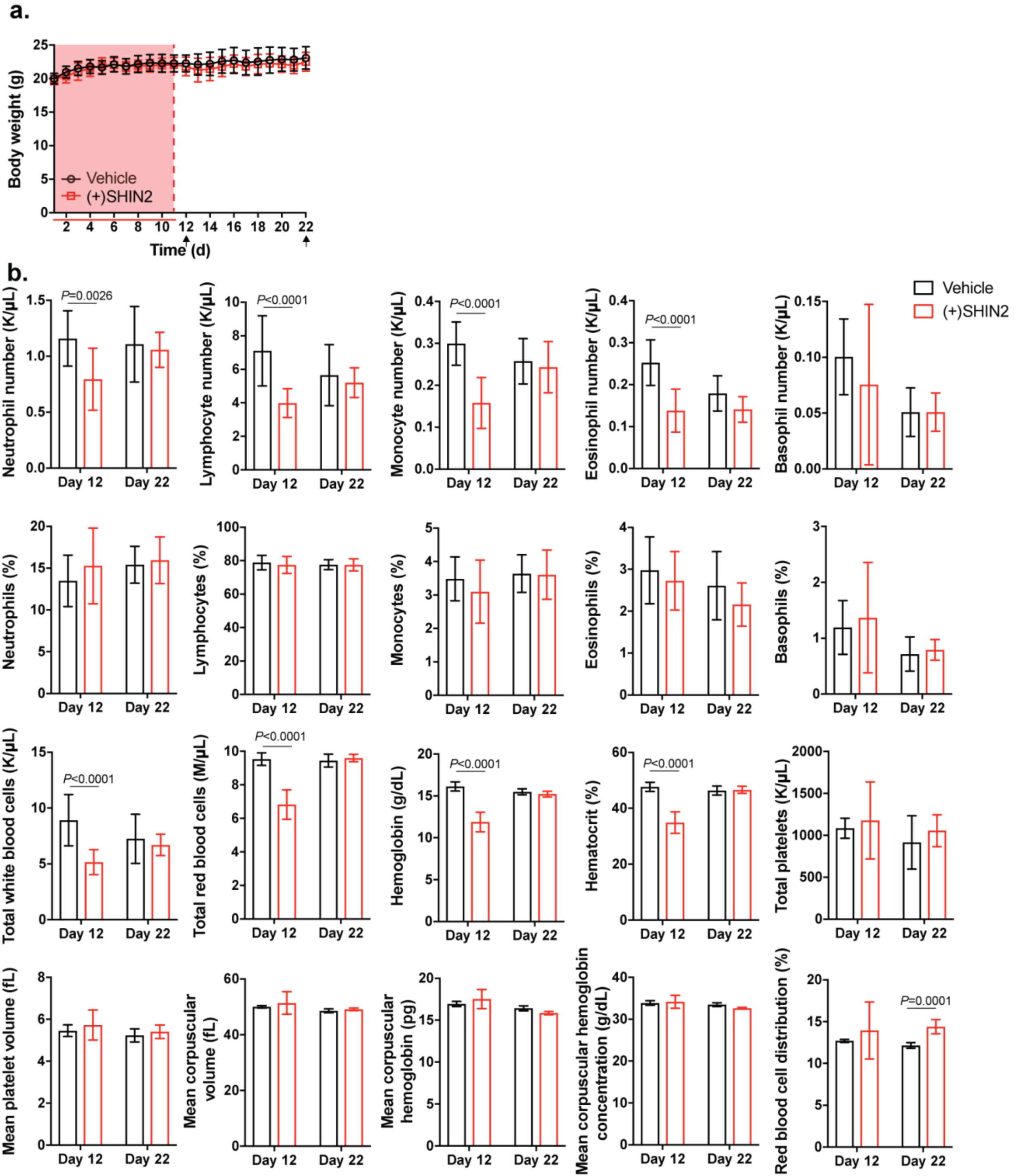


Figure S3, related to Fig.3. SHIN2 treatment leads to reversible heme toxicity. (**a**) Body weight of mice treated with vehicle alone or (+)SHIN2 for 11 consecutive days (highlighted in red) and during 11 days after treatment discontinuation (day 0-12, n=12 per group, day 12-22 n=6 per group). (**b**) Hematological parameters of mice treated with vehicle alone or (+)SHIN2 for 11 consecutive days and 11 days after treatment discontinuation (n=12 per group at day 12; n=6 per group at day 22).*P* values were calculated using an unpaired two-tailed Student's t test.

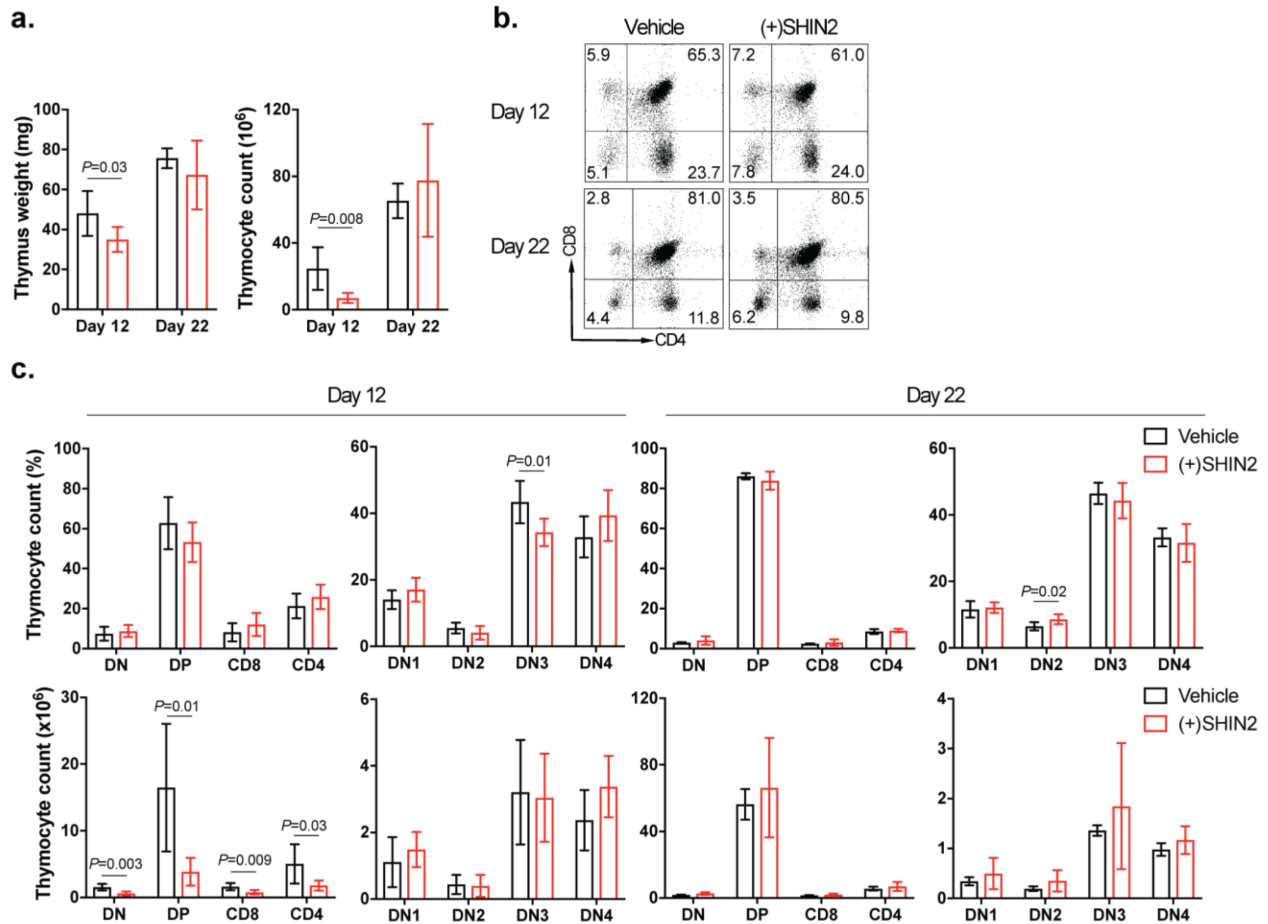


Figure S4, related to Fig.3. SHIN2 treatment leads to reversible effects on T-cell development. (**a**) Thymus weight of mice treated with vehicle alone or (+)SHIN2 for 11 consecutive or 11 days after treatment discontinuation. (**b-c**) Immunophenotypic analyses of thymotyce populations from mice treated with vehicle alone or (+)SHIN2 for 11 consecutive or 11 days after treatment discontinuation. *P* values were calculated using an unpaired two-tailed Student's t test, n=6 per group.

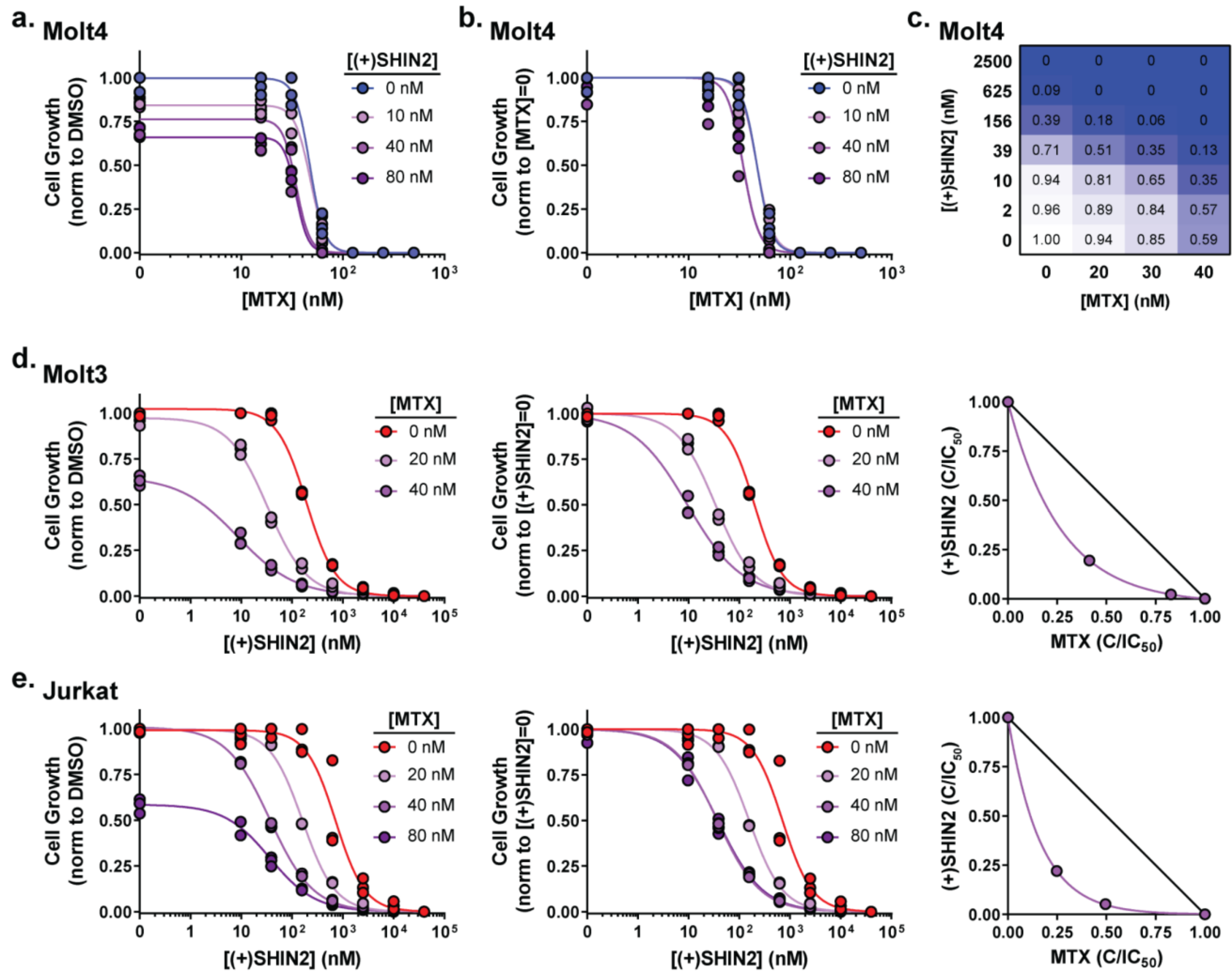


Figure S5, related to Fig.4. SHIN2 and methotrexate synergize in human T-ALL cell lines. (**a-b**) Growth of Molt4 cells incubated with increasing concentrations of methotrexate (MTX) in the presence of 0, 10, 40 and 80 nM (+)SHIN2 normalized to DMSO control proliferation (**a**) or to the same dose of methotrexate without (+)SHIN2 (**b**) (n=3). (**c**) Relative cell growth of Molt4 cells (mean, n=3). (**d-e)** Growth of Molt3 (**d**) and Jurkat (**e**) cells incubated with increasing concentrations of (+)SHIN2 in the presence of the indicated methotrexate concentration normalized to DMSO control proliferation (left, n=3) or to the same dose of methotrexate without (+)SHIN2 (center, n=3), and isobologram for (+)SHIN2 and methotrexate showing the combinations of the drug that achieve a decrease in proliferation > 50%.

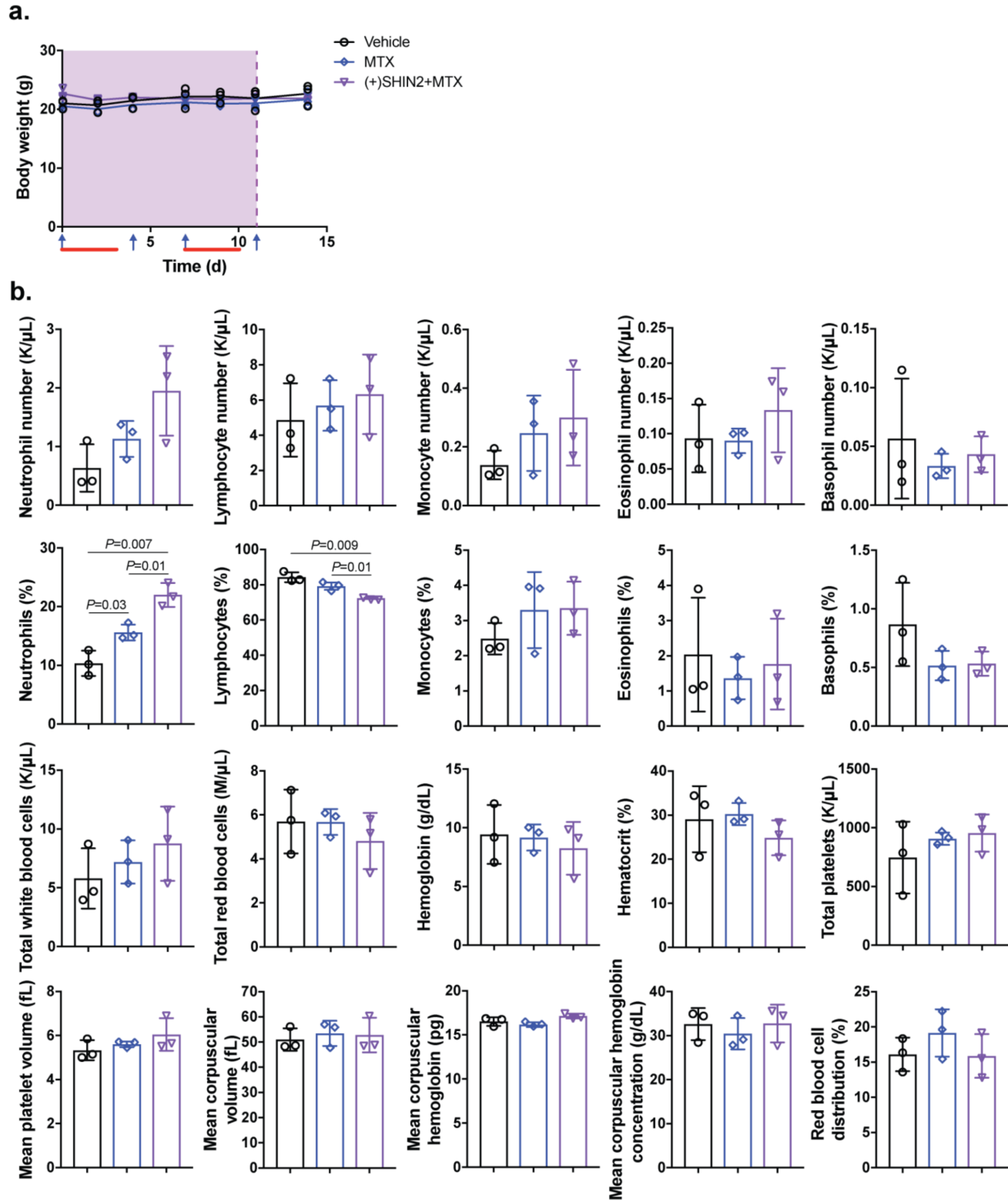


Figure S6, related to Fig.5. Minimal toxicity using SHIN2 and methotrexate combination treatment. (**a**) Body weight of mice treated with vehicle alone, methotrexate or a combination of methotrexate and (+)SHIN2 during two cycles of treatment (highlighted in purple) (n=3 per group). Methotrexate treatment (10 mg/kg) is represented by blue arrows. (+)SHIN2 treatment (200 mg/kg) is represented by red bars. (**b**) Hematological parameters of mice treated with vehicle

alone, methotrexate or a combination of methotrexate and (+)SHIN2 during two cycles of treatment (n=3 per group). Statistical significance was calculated using ANOVA test and the *p* values for paired comparisons were calculated using Tukey's multiple comparisons post-hoc test.

Supplementary References

52 Su X, Lu W, Rabinowitz JD. Metabolite Spectral Accuracy on Orbitraps. *Anal Chem* 2017; **89**: 5940–5948.