Simultaneous inhibition of pan-phosphatidylinositol-3-kinases and MEK as a potential therapeutic strategy in peripheral T-cell lymphomas

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Online Supplementary Design and Methods

Molecular signature and bioinformatic analysis of phosphatidylinositol-3-kinase

The molecular signature of peripheral T-cell lymphoma (PTCL) was identified by comparing the gene expression profile in a series of 38 frozen PTCL cases and six reactive lymph nodes. PTCL cases included: 20 PTCL-not otherwise specified (PTCL-NOS), 15 angioimmunoblastic T-cell lymphoma (AITL) and three anaplastic large cell lymphoma (ALCL: 1 ALCL ALK$^+$ and 2 ALCL ALK$^-$). The research was approved by the ethical committees of the Instituto de Salud Carlos III (Madrid, Spain) and the Hospital Universitario Marqués de Valdecilla (Santander, Spain). The molecular profile, identified by a t-test, was further analyzed using two bioinformatic tools: Connectivity Map (Cmap v2.0, *http://www.broad.mit.edu/cmap*) and Gene Set Enrichment Analysis (GSEA). Cmap was used to identify drugs that might reverse the PTCL gene expression signature. Cmap is a collection of genome-wide transcriptional expression data from a panel of cultured human cell lines (MCF7, PC3 and HL60) treated with a number of small bioactive molecules at several doses. This program calculates an enrichment score based on the similarities between the submitted gene expression signature and gene expression changes induced by the drugs in the panel of cell lines. Taking into account both up-regulated and down-regulated genes, Cmap provides several drugs with a negative enrichment score that indicates the reversion of the PTCL signature. GSEA was used to investigate the correlation between the expression of each phosphatidylinositol-3-kinase (PI3K) isoform and sets of genes grouped according to their cellular functions. We used Biocarta (*http://www.biocarta.com/*), KEGG (*http://www.genome.ad.jp/kegg/*), Ingenuity Pathway Analysis (*http://www.ingenuity.com/*) and other public sources available through the Molecular Signature Database (*http://www.broad.mit.edu/gsea/msigdb/index.jsp*) to generate the gene set database. All microarray data are available at the Gene Expression Omnibus under accession number GSE36172.

Cell lines and primary samples

Six human PTCL and cutaneous T-cell lymphoma (CTCL) cell lines were used. HH (CTCL) and MJ (HTLV1$^+$ PTCL) were obtained from the American Type Cell Collection (ATCC, Rockville, MD, USA); MyLa (mycosis fungoides) and HuT78 (Sézary's syndrome) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK); SR786 (ALCL ALK$^+$) and DERL7 (hepatosplenic γδ T-cell lymphoma) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All of the cells except those of the MJ cell line, were cultured in RPMI 1640 medium (IMDM medium for MJ cells) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (all from Invitrogen Corporation, Carlsbad, CA, USA) in a humidified atmosphere at 37°C with 5% CO_2. The DERL7 cell line was supplemented with 20% fetal bovine serum and 20 ng/mL human interleukin-2 (PeproTech, Rocky Hill, NJ, USA). All cell lines were previously authenticated by the DSMZ.

Primary samples were used to measure the basal *PIK3CD* and *PIK3CA* mRNA levels. Tumor and normal T cells were isolated from the peripheral blood of five patients with Sèzary's syndrome and three healthy donors, respectively, through negative selection using the RosetteSep kit (StemCell Technologies, Grenoble, France). Sample purity was checked by flow cytometry, and an enrichment of >90% CD3$^+$ cells was ensured in all samples. Additionally, the drug sensitivity of primary T cells from eight patients with CTCL (5 with Sézary's syndrome and 3 with mycosis fungoides) was tested.

Sequencing of relevant phosphatidylinositol-3-kinase pathway and related genes in peripheral T-cell lymphoma cell lines and cases

DNA from six PTCL cell lines was extracted following standard procedures. DNA yields and purity were determined by measuring absorbance at 260/280 nm. DNA was amplified by high-resolution melting using the primers shown in *Online Supplementary Table S1*. Briefly, each amplification reaction was performed using LightScanner High Sensitivity Master Mix (Idaho Technology, Salt Lake City, UT, USA) in a LightCycler® 480 (Roche, Basel, Switzerland), following the manufacturer's instructions. The polymerase chain reaction (PCR) conditions consisted of an initial denaturalization step at 95°C for 10 min, followed by 45 cycles at 94°C for 20 s and 60°C for 30 s. The temperature was then rapidly decreased to 40°C over 1 min to allow the formation of heteroduplexes, followed by a continuous increase of the temperature up to 95°C at a rate of 0.02°C and 25 fluorescence acquisitions per second to build the melting curves. Finally, samples were grouped, taking into account the melting profile of the amplified sequences using the LightCycler®480 software 1.5.0 sp3 (Roche).

The *PIK3CA* and *PIK3CD* genes were further analyzed in the panel of six PTCL cell lines and a series of 27 frozen PTCL cases (13 PTCL-NOS, 10 AITL, 3 ALCL ALK$^+$, 1 ALCL ALK$^-$) by direct sequencing. Briefly, the PCR was performed in a 50 μL reaction containing 250 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, and 1 unit of Platinum Taq polymerase (Invitrogen Life Technologies). The PCR consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 59°C for 45 s, 72°C for 30 s and a final step at 72°C for 5 min. Amplified products were visualized on a 2% agarose gel to check for the expected amplicon lengths. Sequencing reactions were done using BigDye Terminator v3.1 (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions and analyzed with a 3130xl Genetic Analyzer (Applied Biosystems). Sequence files were edited using SeqScape v2.5 (Applied Biosystems).

PIK3CD *and* PIK3CA *genetic silencing*

MyLa, SR786 and HuT78 cell lines were electroporated with specific small interfering RNA (siRNA) against the *PIK3CD* and *PIK3CA* genes, using the Neon® Transfection System (Invitrogen) according to the manufacturer's instructions. Briefly, cells were incubated without antibiotics overnight and resuspended in buffer R at 500,000 cells/mL. Then, siPIK3CD (#s10530) or siPIK3CA (#s10521, both from Invitrogen) and the non-template control (NTC, AM4635, Ambion) were added to the cells at a final concentration of 100 nM. Microporation conditions were set up for each cell line, aiming for the highest transfection efficiency with the minimum loss of cell viability. These conditions were: 1300 V, 20 ms and two pulses for MyLa; 900 V, 30 ms and two pulses for SR786; and 1150 V, 20 ms and two pulses for HuT78. Cells were then electroporated in these conditions to allow the entry of the siRNA into the cell and 100 μL of the suspension were seeded in 2 mL for 24, 48, 72 and 96 h.

RNA extraction and quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted and purified using the RNeasy Mini-Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions in order to check the *PIK3CD/PIK3CA* knockdown efficiency and to measure basal mRNA levels of these genes in PTCL cell lines, primary tumor T cells and normal T cells.

The expression of *PIK3CD* and *PIK3CA* was measured by quantitative reverse transcriptase PCR. Briefly, total RNA was retrotranscribed using the SuperScript enzyme (Invitrogen) (10 min at 25°C, 60 min at 42°C and 15 min at 70°C). Two microliters of the resulting cDNA were placed in a 384-well plate with 0.75 μL Taqman probes (*PIK3CD* Hs00192399_m1; *PIK3CA* Hs00180679_m1; and the endogenous control *RN18S1* Hs03928990_g1; all from Applied Biosystems) in a final volume of 15 μL. The PCR amplification was performed using Applied Biosystems Prism 7900HT Sequence Detection System under the following thermal cycler conditions: 2 min at 50°C, 10 min at 95°C and 30 cycles (15 s at 95°C and 1 min at 60°C).

Cell viability assay

For drug cytotoxicity experiments, PTCL cell lines were seeded in 96-well plates at a density of 10,000 cells per well, and PI3K inhibitors (PI3Ki), MEK inhibitors (MEKi) or their combinations were added at a range of doses for 72 h, using dimethylsulfoxide (DMSO) as a control. Primary tumor T cells were treated for 48 h. Cell viability was measured as the intracellular ATP content using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. All experiments were done in triplicate and all numerical data were expressed as the average of the values ± the standard error of the mean (SEM).

Drug combination assay

For drug combination experiments, PTCL cell lines and primary tumor T cells were seeded in 96-well plates at a density of 10,000 cells per well, and a PI3Ki + MEKi combination was added for 48 h or 72 h, using DMSO as a control. Cells were treated with a wide range of doses and cell viability was measured as explained above. The combination index (CI) was calculated using CalcuSyn software (Biosoft, Ferguson, MO, USA) following the method of Chou and Talalay, where CI<1, ≈1, and >1 indicate synergism, additive effect and antagonism, respectively. All experiments were done in triplicate and all numerical data are expressed as the average of the values ± SEM.

Flow cytometry analysis

The distribution of cells among different phases of the cell cycle and induction of apoptosis were evaluated by flow cytometry using propidium iodide (PI, Sigma-Aldrich, St Louis, MO, USA) staining and the APC-annexin V (Beckton Dickinson, BD, Franklin Lakes, NJ, USA) binding assay, respectively. All data from 10,000 cells were detected on a FACSCalibur flow cytometer (BD) and analyzed using CellQuest Pro software (BD).

The levels of pAKT(Ser473) and total AKT were measured by flow cytometry in all cell lines after GDC-0941 treatment. Briefly, cells were fixed and incubated with pAKT(Ser473) and AKT antibodies (#9271 and 9272, Cell Signaling Technology, Inc., Danvers, MA, USA). They were then incubated with secondary Alexa Fluor 647 antibody (A21244, Invitrogen) and

measured by flow cytometry.

All experiments were done in triplicate and the statistical significance of differences between groups was determined by Student's independent samples t-test (SPSS v17.0).

Protein extraction, antibodies and western blot

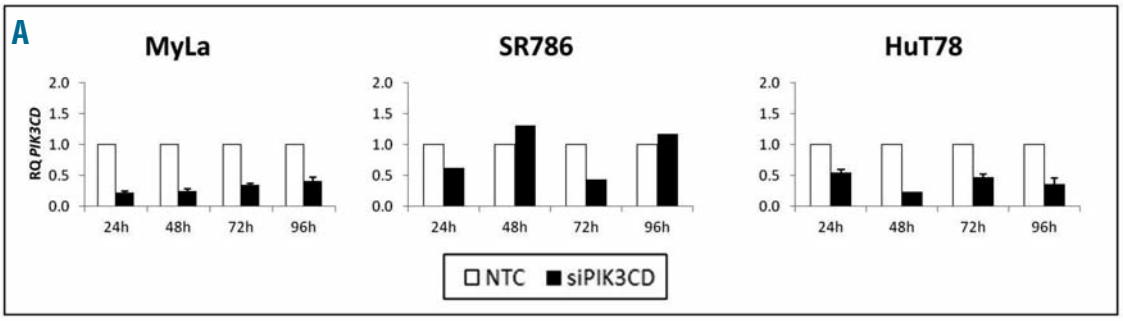
Cells untreated and treated as indicated were harvested and washed twice in phosphate-buffered saline for protein extraction. Briefly, cells were lysed using RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Roche) for 30 min on ice and centrifuged. The protein fraction in the supernatant was then recovered and quantified using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Inc., Hercules, CA, USA), in accordance with the manufacturer's recommendation.

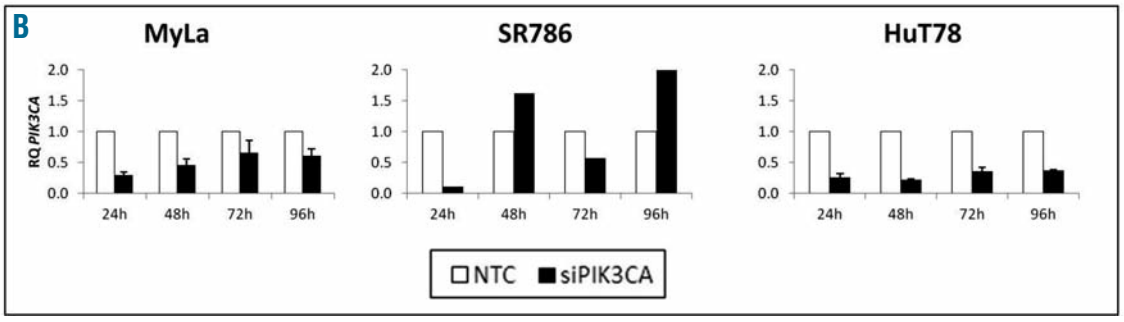
For western blot, 60 μg of proteins were resolved by sodium dodecyl polyacrylamide gel electrophoresis in a 12% gel and transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). Membranes were blocked in 5% bovine serum albumin and incubated with primary antibodies. They were then incubated with secondary antibodies (Alexa 680 nm or Alexa 800 nm, Rockland, Gilbertsville, PA, USA) and scanned with an Odyssey Infrared System Scanner (LI-COR, Biosciences, Lincoln, NE, USA). The primary antibodies for western blots were obtained as follows: total GSK3β (ab73271 from Abcam Inc., Cambridge, MA, USA), phospho-GSK3α(S21)/β(S9) (#9331), phospho-p70S6K(T389) (#9206), total p70S6K (#9202), phospho-ERK1(Thr202)/pERK2(Tyr204) (#9101) and total ERK1/2 (#9102) from Cell Signaling Technology Inc. and α-tubulin (T-6074) from Sigma-Aldrich.

Online Supplementary Table S1. Primers used to sequence several genes involved in the PI3K pathway.

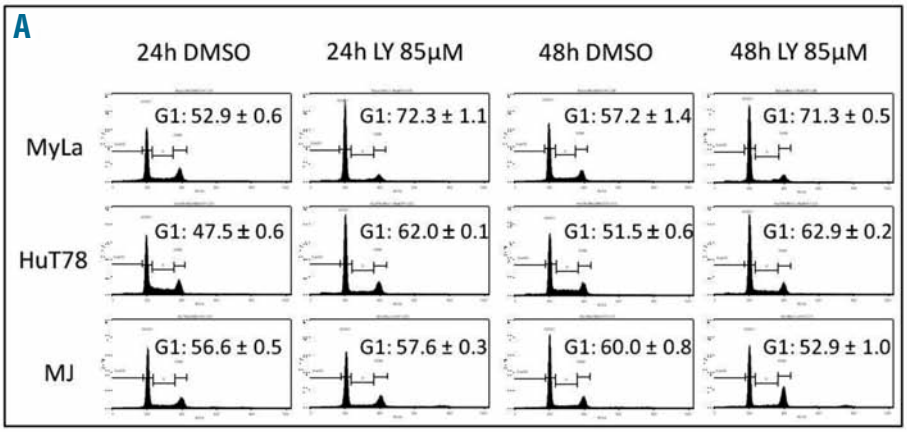
Gene (exon)	5'-3' sequence
AKT1 (4F)	CTGGCCCTAAGAAACAGCTCC
AKT1 (4R)	CGCCACAGAGAAGTTGTTGA
BRAF (15F)	TGCTTGCTCTGATAGGAAAATG
BRAF (15R)	AGCATCTCAGGGCCAAAAAT
KRAS (2F)	GGTGGAGTATTTGATAGTGTATTAACC
KRAS (2R)	AGAATGGTCCTGCACCAGTAA
NRAS (3F)	GGTGAAACCTGTTTGTTGGA
NRAS (3R)	TATGTGTCTCCTTCGGAAGC
PIK3CA (20F)	TGGGGTAAAGGGAATCAAAAG
PIK3CA (20R)	CCTATGCAATCGGTCTTTGC
PIK3CA (9F)	GATTGGTTCTTTCCTGTCTCTG
PIK3CA (9R)	CCACAAATATCAATTTACAACCATTG
PIK3CD (18F)	CCCAAGCCCCGCCACAAG
PIK3CD (18R)	CCCAAGCCCTTAAGTCCC
PIK3CD (5F)	GCAAAGGTAGCTCTGCCG
PIK3CD (5R)	GGGCTTGCGGATGACTGA
PIK3CD (8F)	TCACGGGCCTCACCATAG
PIK3CD (8R)	TCCTCTGCTACCCCTCCA
PTEN (1F)	CATCTCTCTCCTCCTTTT
PTEN (1R)	ATGTTATAGTCACCCAAACTA
PTEN (2F)	ATAAATACATTGACCACCTT
PTEN (2R)	AAATCAAAGCATTCTTACC
PTEN (3F)	AGAAAATCTGTCTTTTGGT
PTEN (3R)	AAATGTATCTTTAACTCTACCTC
PTEN (4F)	GGCAATGTTTGTTAGTATTAG
PTEN (4R)	ATATGTATCTCACTCGATAATCT
PTEN (5Fa)	ACATTTCTAAAGTTACCTACTTG
PTEN (5Fb)	GCTAAGTGAAGATGACAATC
PTEN (5Ra)	CACATATCATTACACCAGTTC
PTEN (5Rb)	CTGTTTTCCAATAAATTCTC
PTEN (6F)	TACCATAGCAATTTAGTGAA
PTEN (6R)	ATGAGAATTTCAAGCACTTA
PTEN (7F)	TCCATATTTCGTGTATATTG
PTEN (7R)	CAATGAAAGTAAAGTACAAACC
PTEN (8Fa)	GTTTAACATAGGTGACAGATT
PTEN (8Fb)	AAGTCTATGTGATCAAGAAATC
PTEN (8Ra)	ATTATCTGCACGCTCTATAC
PTEN (8Rb)	TACTCCTAGAATTAAACACACAT
PTEN (9F)	GAGTCATATTTGTGGGTTT
PTEN (9R)	GTGTTTTATCCCTCTTGATA

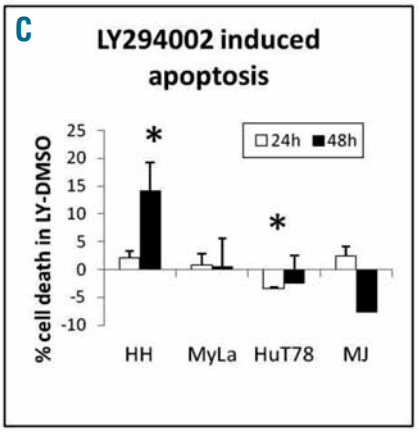
Known mutations in indicated exons in AKT1, BRAF, KRAS, NRAS, PIK3CA, PIK3CD *and* PTEN *genes were investigated in six PTCL and CTCL cell lines.* PIK3CD *sequences were also studied in 27 PTCL cases. (F: forward primer; R: reverse primer).*

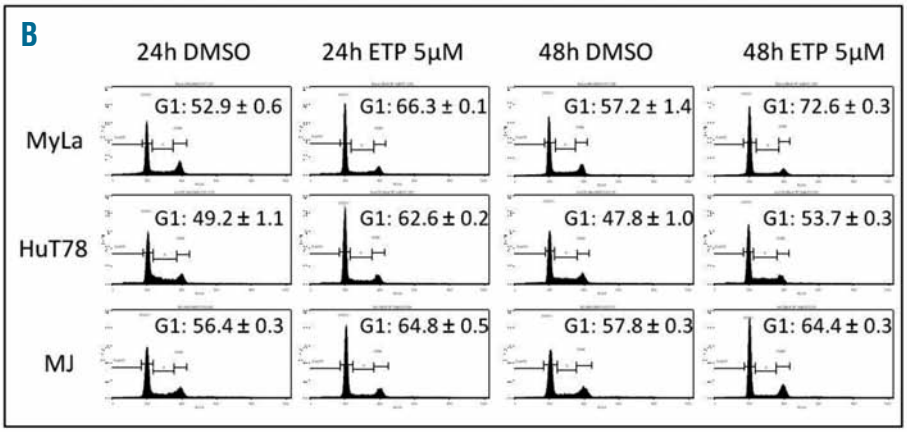


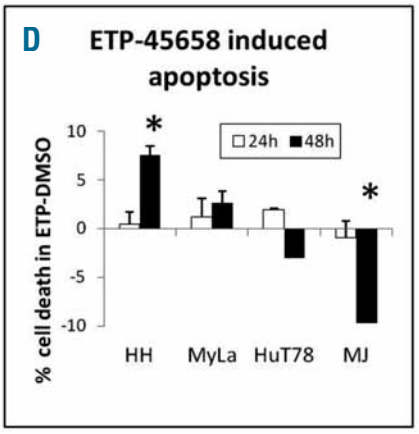


Online Supplementary Figure S1. Knockdown efficiency of *PIK3CD* and *PIK3CA* genes at the mRNA level. PTCL cell lines were electroporated with (A) 100 nM siPIK3CD and (B) 100 nM siPIK3CA, and RNA levels of each of the genes were measured by quantitative RT-PCR. The Y axis represents the relative quantification (RQ) of RNA in silenced cells compared to the non-template control (NTC)-electroporated cells.

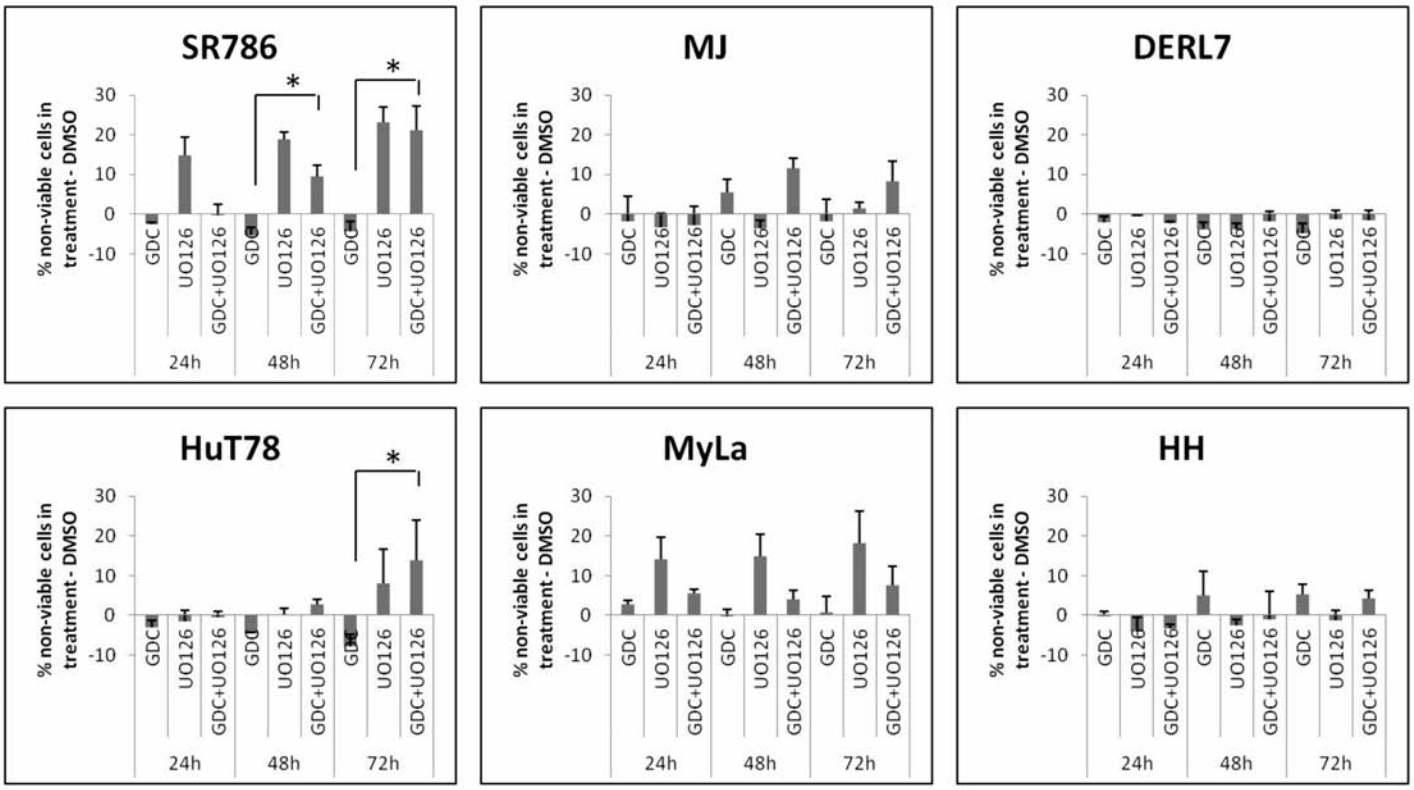




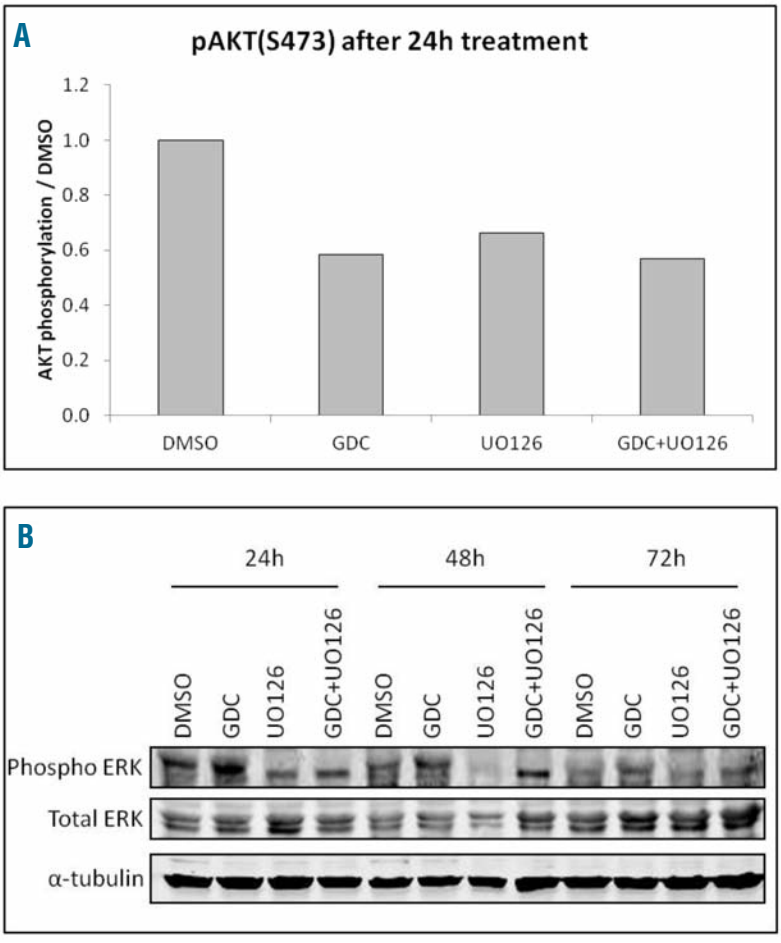




Online Supplementary Figure S2. Effects of other pharmacological pan-PI3Ki on PTCL cell line survival. All the cell lines were arrested at G1 phase of the cell cycle in a dose- and time-dependent manner upon treatment with (A) LY294002 and (B) ETP-45658. Only GDC-0941 sensitive cell lines underwent apoptosis upon treatment with (C) LY294002 and (D) ETP-45658. The Y axis indicates the percentage of annexin V^+/propidium $iodide^-$ plus annexin V^+/propidium $iodide^+$ cells after treatment with 17 μM LY294002 or 5 μM ETP-45658 minus the cell death in DMSO. *Indicates a statistically significant difference compared with DMSO-treated cells (P<0.05).



Online Supplementary Figure S3. Apoptosis induced by the combination of PI3Ki + MEKi in PTCL cell lines. PTCL cell lines were treated with 1 μM GDC-0941, 10 μM UO126 and their combination for 24, 48 and 72 h. The Y axis indicates the percentage of annexin V$^+$/propidium iodide$^-$ plus annexin V$^+$/propidium iodide$^+$ cells after treatment minus the cell death in DMSO. Overall, the combination did not enhance the apoptosis induced by each drug alone; only in SR786 and HuT78 cell lines were both drugs significantly more effective than GDC-0941 alone (although no more than UO126 alone). (*P<0.05).



Online Supplementary Figure S4. pAKT and pERK levels in SR786 cells treated with a combination of PI3Ki + MEKi. The SR786 cell line was treated with 1 μM GDC-0941, 10 μM UO126 and their combination for 24, 48 and 72 h, and (A) AKT and (B) ERK phosphorylation were measured by flow cytometry and western blot, respectively.