ARTICLE - Non-Hodgkin Lymphoma

Selective pharmacologic targeting of CTPS1 shows single-agent activity and synergizes with BCL2 inhibition in aggressive mantle cell lymphoma

Romane Durand,[1] Céline Bellanger,[1] Charlotte Kervoëlen,[1] Benoit Tessoulin,[2] Christelle Dousset,[1] Emmanuelle Menoret,[1] Hélène Asnagli,[3] Andrew Parker,[3] Philip Beer,[3] Catherine Pellat-Deceunynck[1] and David Chiron[1]

[1]Nantes Université, INSERM, CNRS, Université d'Angers, CRCI2NA, Nantes; [2]Nantes Université, CHU de Nantes, INSERM, CNRS, Université d'Angers, CRCI2NA, Nantes and [3]Step Pharma, Saint-Genis-Pouilly, France

Correspondence: D. Chiron
david.chiron@univ-nantes.fr

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This supplementary file contains supplementary Methods, supplementary references, supplementary Table S1, supplementary Figures S1-6 and associated legends.

Supplemental Methods

Cell line source

MINO, REC-1, MAVER-1 and GRANTA-519 were purchased from DSMZ (Germany). Z138, JeKo-1 and JVM2 from ATCC (USA). UPN1, HBL2 and SP53 were kindly provided by Prof. V. Ribrag (Institut Gustave Roussy, France), Prof. M. Callanan (University of Burgundy, France) and Prof. S. Chen-Kiang (Cornell University, NY), respectively. NTS3 cell line has been generated in our laboratory (characterized by GEP, GSE86322). Resistant cell lines were generated using *in vitro* selection and named -VenR and -IbruR for venetoclax and ibrutinib resistance, respectively. HLA-A, -B, and -C typing was carried out by Next-Generation-Sequencing (NGS) using Omixon Holotype HLA® (Omixon, Budapest, Hungary, EFS Nantes).

Reagents and antibodies

STP-B, a highly selective inhibitor of CTPS1[1], was provided by Step Pharma (Saint-Genis-Pouilly, France). Venetoclax and ibrutinib were obtained from Selleck Chemicals. Anti-CD19 was purchased from Beckman Coulter, AnnexinV was from Immunotools. For immunoblotting, anti-CTPS1 and anti-CTPS2 pAbs were from Atlas Antibodies. Anti-MCL1 mAb (D35A5), anti-phospho(Ser65) 4E-BP1 mAb (174A9) and anti-4E-BP1 pAb were from Cell Signaling. Anti-BCL2 mAb (clone 7) was from BD Biosciences. Anti-BCL-XL mAb (D-3), anti-Cyclin D1 pAb (H-295) and anti-Cyclin D2 pAb (C-17) were from Santa Cruz Biotechnology. Anti-BIRC5 pAb was from R&D Systems and anti-Puromycin mAb (12D10) was from Sigma-Aldrich.

BH3 profiling assay

BH3 profiling assay was performed in MCL cell lines as previously described[2,3]. Z138 cells were pre-treated or not with 50 or 500 nM STP-B for 24 hours. Cells were permeabilized in DTEB buffer with 0.002% digitonin and exposed to 10 or 20 µM of the BCL2 specific BH3-mimetic venetoclax or DMSO (control condition) for 1 hour at 27°C, before fixation with 2% formaldehyde at room temperature for 15 min. After addition of neutralizing buffer (Tris/Glycine

buffer) for 5 min, cells were stained with anti-cytochrome c antibody (BD Biosciences) in 0.1%Saponin/1%BSA/PBS overnight at 4°C. Loss of cytochrome c was analyzed by flow cytometry by gating the cytochrome c positive population.

Puromycin incorporation assay

Puromycin incorporation (SUnSET) assay was performed using a previously established protocol[4]. Briefly, cells were pre-treated with 50 or 500 nM STP-B for 24 hours, before addition of 1 µM puromycin for 30 min. Cells were washed twice with ice-cold PBS before standard protein extraction. Puromycin incorporation was revealed by western blotting using anti-puromycin antibody diluted at 1:10000.

Animal models

Z138-xenograft subcutaneous model

Z138, an ibrutinib/venetoclax double resistant blastoid MCL cell line, was selected to evaluate the *in vivo* efficacy of STP-B alone or combined with venetoclax. Z138 cells ($5x10^6$) were injected subcutaneously into the right flank of mice. Tumor growth was measured with calipers three times a week and mice were randomized when the tumor reached approximately 50 to 150 mm^3. STP-B was dosed at 30 mg/kg/day subcutaneously days 1-4 of a 7-day cycle and venetoclax at 75 mg/kg/day orally days 2-5 of a 7-day cycle for three cycles.

MCL Patient Derived Xenograft (PDX) disseminated model

In vivo efficacy of STP-B was also evaluated using an ibrutinib-refractory *TP53*^{MUT} MCL PDX disseminated model. PDX MCL cells ($1x10^6$) were injected intravenously into the tail vein of NSG conditioned with sublethal (1.5 Gy) total body irradiation. Treatment was initiated 1 week after transplantation: STP-B was dosed at 30 mg/kg/day subcutaneously days 1-4 of a 7-day cycle for three cycles. At weeks 2 and 3 post-injection, blood sample from each mouse was taken by tail vein puncture in heparin tube and percentage of MCL cells in the peripheral blood was measured by flow cytometry using human CD45 staining.

Supplemental References

1. Asnagli H, Minet N, Pfeiffer C, et al. CTP Synthase 1 Is a Novel Therapeutic Target in Lymphoma. *HemaSphere*. 2023;7(4):.
2. Chiron D, Bellanger C, Papin A, et al. Rational targeted therapies to overcome microenvironment-dependent expansion of mantle cell lymphoma. *Blood*. 2016;128(24):2808–2818.
3. Decombis S, Bellanger C, Le Bris Y, et al. CARD11 gain of function upregulates BCL2A1 and promotes resistance to targeted therapies combination in B-cell lymphoma. *Blood Journal*. 2023;blood.2023020211.
4. Ravi V, Jain A, Mishra S, Sundaresan NR. Measuring Protein Synthesis in Cultured Cells and Mouse Tissues Using the Non-radioactive SUnSET Assay. *CP Molecular Biology*. 2020;133(1):e127.

STP	**IC50 values (µM)**		RNA-Seq (log2)*		TP53	Other Drug responses	
MCL cell lines	Mean	SEM	*CTPS1*	*CTPS2*	STATUS	Venetoclax	Ibrutinib
Z138_WT	0.015	0.005	*ND*	*ND*	WT	R	R
Z138 p53 KO1	0.014	0.003	*ND*	*ND*	**KO**	R	R
Z138 p53 KO2	0.011	0.002	*ND*	*ND*	**KO**	R	R
JEKO	0.036	0.001	1,0	1,0	Mutated	R	S
JEKO-IbruR	0.028	0.002	1,0	1,0	Mutated	R	**R**
MAVER	0.22	0.07	1,0	1,0	Mutated	S	R
MAVER-VenR	0.29	0.12	1,0	0,9	Mutated	**R**	R
MINO	> 3.0	-	1,0	1,0	Mutated	S	S
MINO-VenR	> 3.0	-	1,0	1,0	Mutated	**R**	S
MINO-IbruR	> 3.0	-	0,9	1,0	Mutated	S	**R**

Table S1: Isogenic resistant cell lines characteristics.
CTPS1/2 levels are relative to the parental cell line. R: resistant (IC_{50} > 1000nM), S: sensitive (IC_{50} < 1000nM), ND: not determined

Supplemental Figure Legends

Figure S1: B-cell lymphoma cells relies on CTPS1 to proliferate.

A: Immunoblotting of CTPS1 and CTPS2 was performed in 6 MCL cell lines. Both anti-CTPS1 and anti-CTPS2 antibodies (Atlas Antibodies) were used at a dilution of 1:1000. Secondary antibody (goat anti-rabbit) was used at a dilution of 1:5000. Exposure times were 4 and 20 seconds for CTPS1 and CTPS2 respectively. Protein levels relative to GAPDH level are indicated.

B: Dependence to *CTPS1* and *CTPS2* was evaluated in 70 B-cell lymphoma cell lines using the DepMap CRISPR dataset.

Figure S2: Sensitivity to STP-B is independent of *CTPS1/2* expression, resistance to BTK/BCL2 targeted therapies and *TP53* status.

A: *CTPS1/2* expression from 3'SRP data was compared with STP-B IC_{50} values in 10 MCL cell lines. Spearman test was used.

B: STP-B IC_{50} values were compared in MCL cell lines depending on *TP53* status, sensitivity (S) or resistance (R) to venetoclax and ibrutinib (see Table 1 for more details). Mann-Whitney test was used.

C: Resistance of Jeko-IbruR and Maver-VenR sublines was validated by CTG assay and Z138 $TP53^{KO}$ clones confirmed their resistance to Nutlin3a-induced cell death.

Figure S3: STP-B is well tolerated in mouse models

A: The body weight variation of (n=5) control and (n=5) treated mice was evaluated upon STP-B (30 mg/kg) treatment.

B: Mouse PBMC count was determined by flow cytometry (huCD45-negative count) 21 days and 28 days after engraftment. Mann-Whitney test was used.

Figure S4: Transcriptomic analysis of MCL cells was performed in response to STP-B.

A: The heat-map highlights the transcriptional clustering of 9 MCL cell lines treated (STP-B) or not (CT) with STP-B IC_{50} for 24 hours.

B: The volcano-plot represents the differentially expressed (DE) genes upon STP-B treatment in 9 MCL cell lines. Dotted lines indicate the cut-off for significance (Log_2 FC < -0.5 or > 0.5 and adjusted p-value < 0.05). The greatest regulated genes (red dots) are annotated on the graph.

Figure S5: CTPS1 inhibition synergizes with venetoclax

A: STP-B and ibrutinib combination efficacy was evaluated in 9 MCL cell lines. Cells were treated for 72 hours with 3 concentrations of STP-B and ibrutinib as indicated. Combination inhibitory effect was assessed by CTG assay. Red: most synergistic area, green: lowest synergistic area. Bliss synergy score is indicated.

B: STP-B and venetoclax combination efficacy was evaluated in 9 MCL cell lines. Cells were treated for 72 hours with 3 concentrations of STP-B and venetoclax as indicated in each graph. Cell death was determined by Annexin-V staining.

Figure S6: CTPS1 inhibition led to translation inhibition and consequent decrease of MCL1 protein level.

A: MCL1 mRNA and protein levels were determined by quantitative PCR and immunoblotting following treatment with 50 or 500 nM STP-B for 24 hours in Z138 and JeKo-1. Each graph represents 3 independent experiments.

B: CCND1, CCND2 and BIRC5 protein expression was determined by immunoblotting upon STP-B treatment.

C: CCND1, CCND2 and BIRC5 protein levels were normalized to GAPDH levels and gene expression was assessed by 3'SRP upon STP-B treatment.

D: Phospho(Ser65)-4E-BP1 and 4E-BP1 protein levels were assessed in Z138 and JeKo-1 cells treated for 24 hours with STP-B. Protein levels relative to tubulin level are indicated.

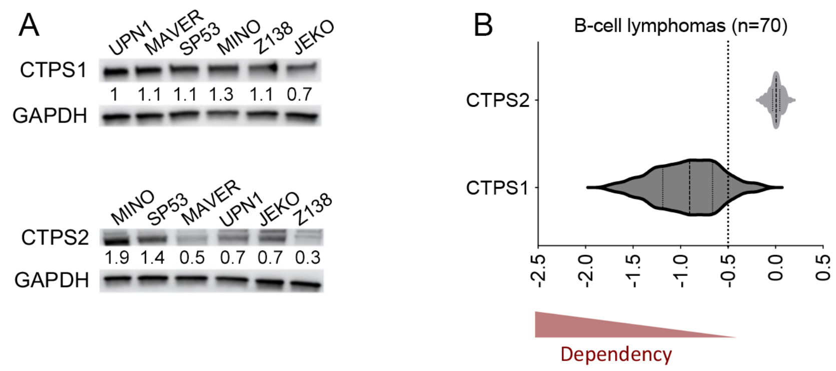


Figure S1

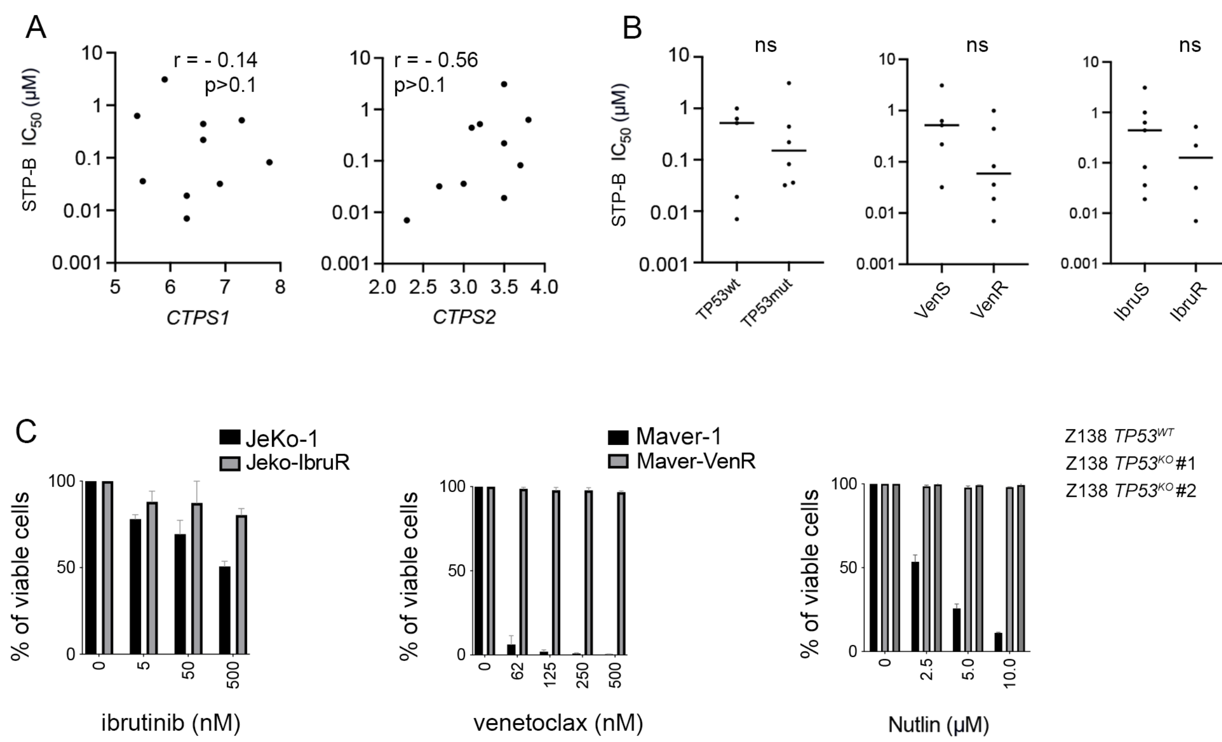
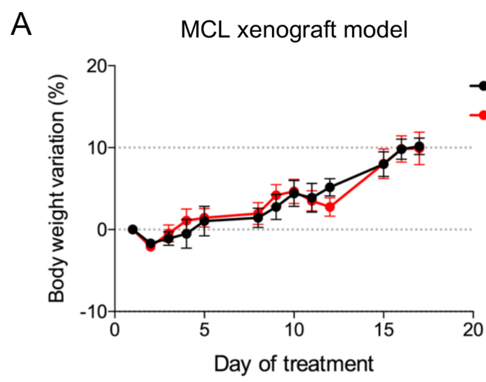


Figure S2



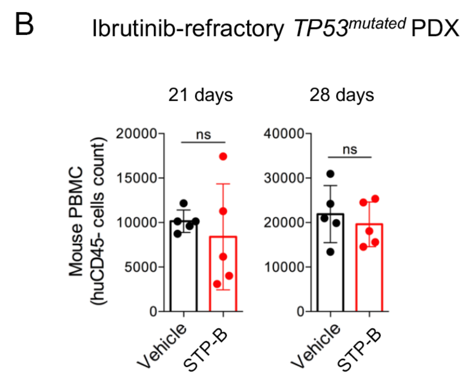
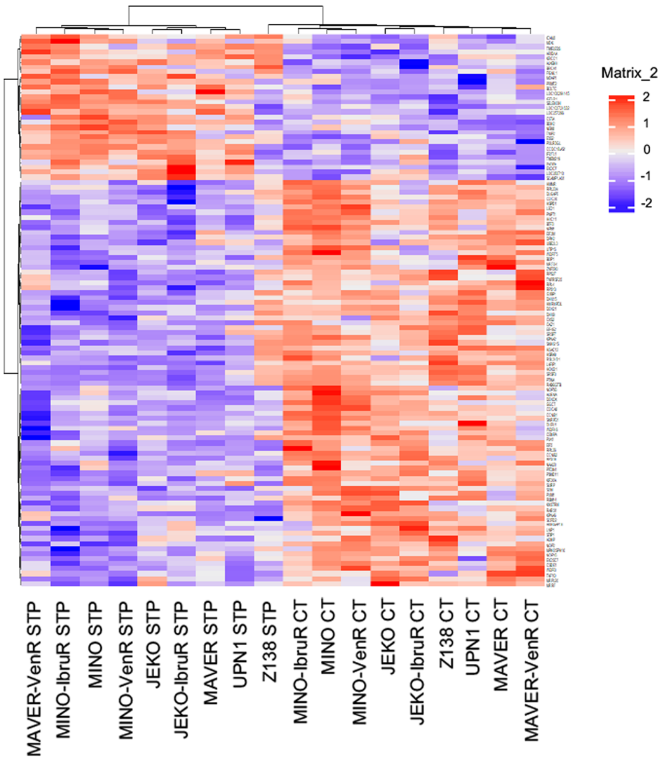


Figure S3

A



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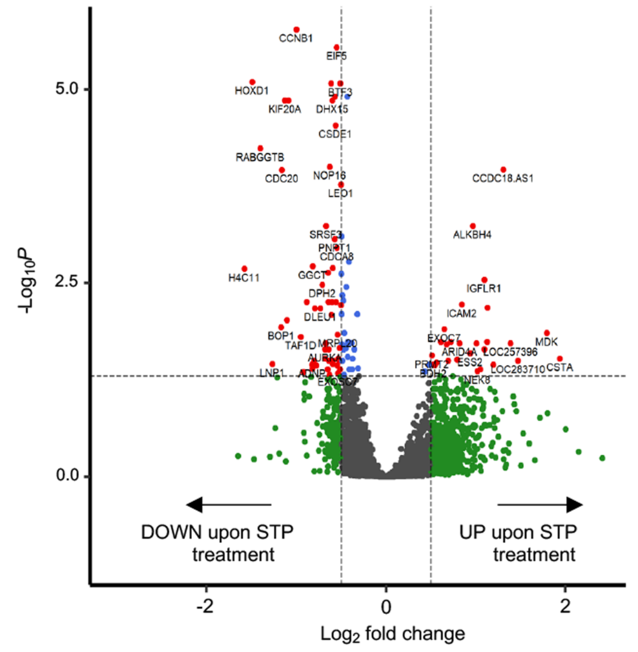

Figure S4

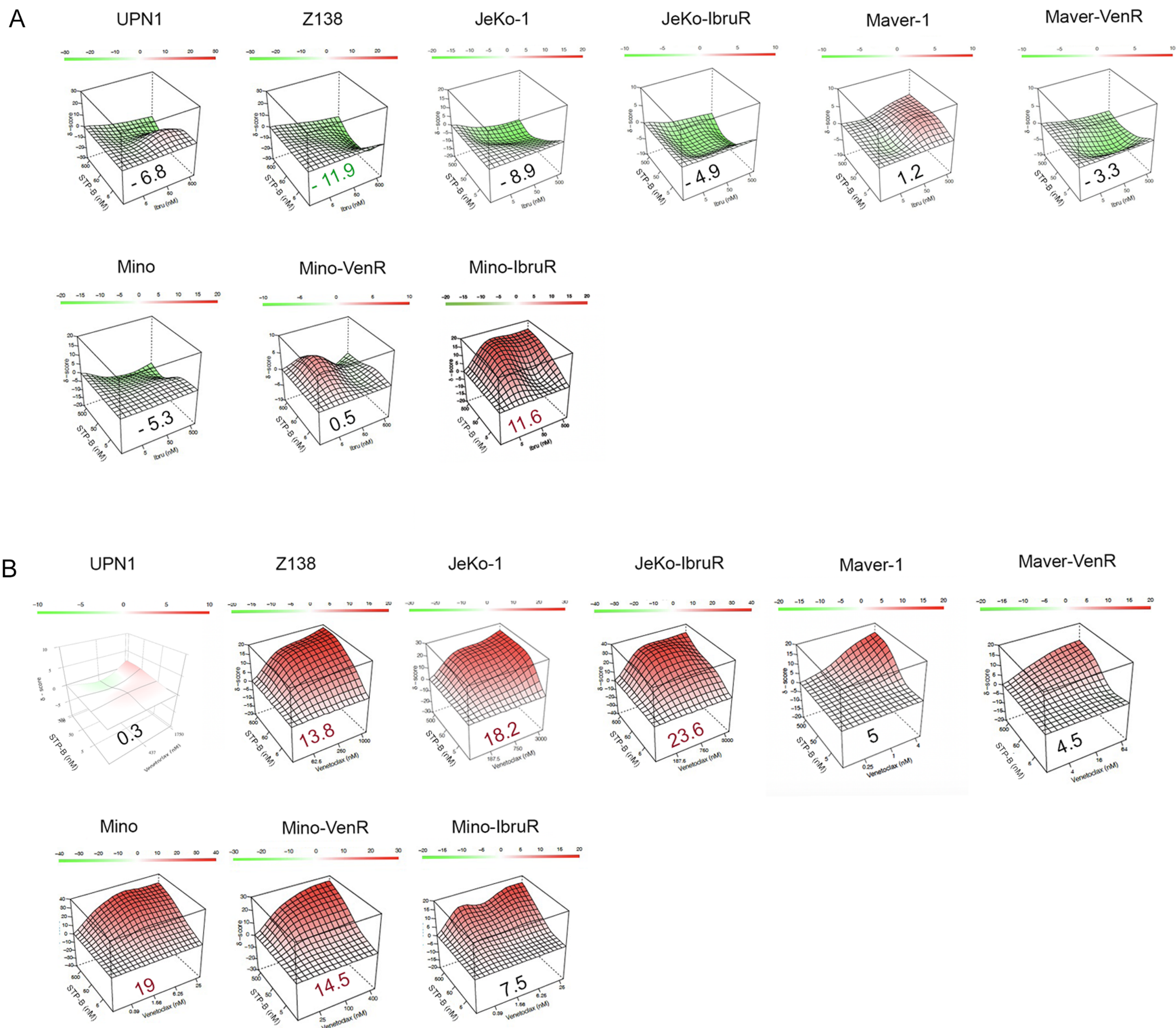

Figure S5

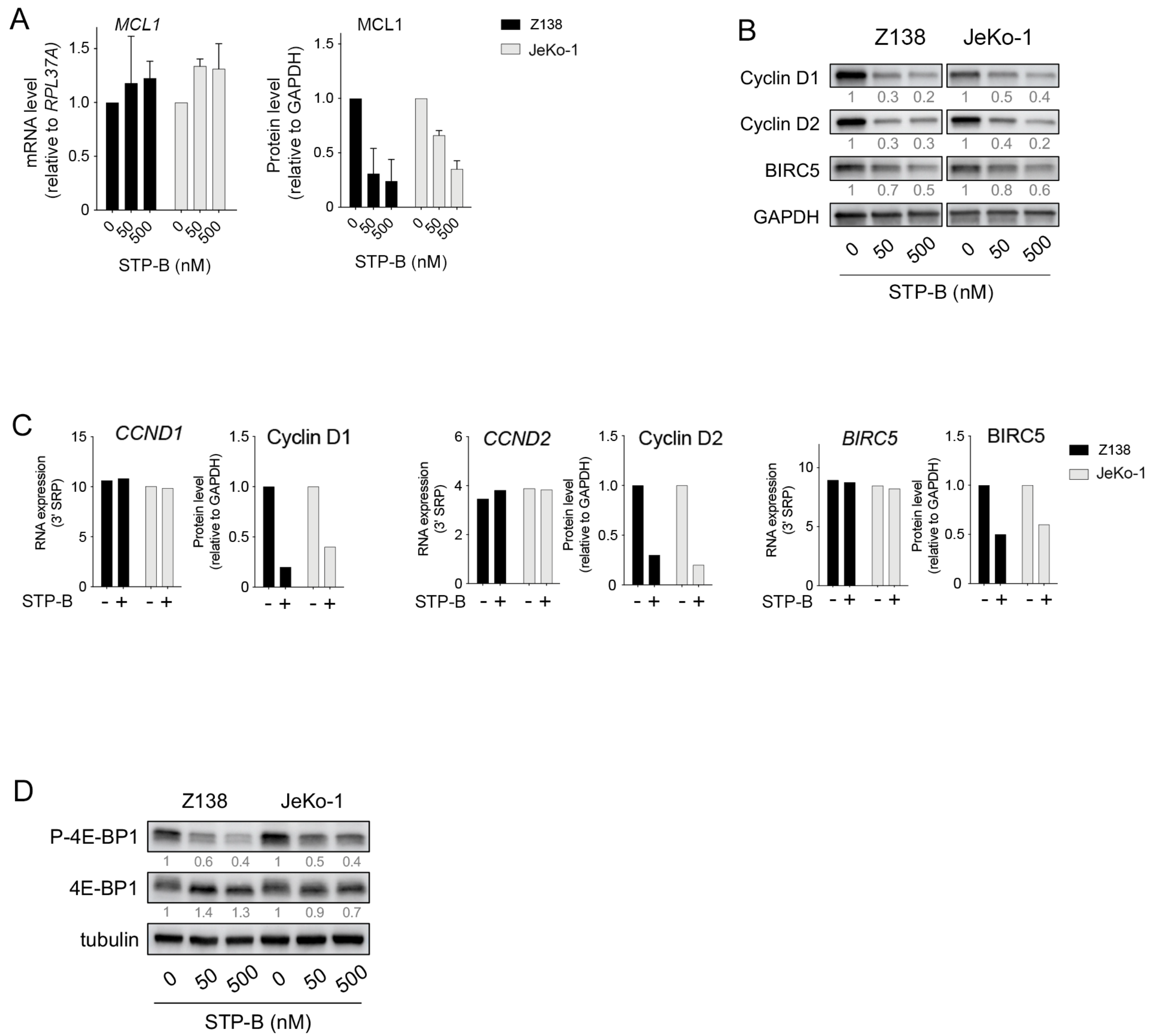

Figure S6