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

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Abstract

Innovative therapeutic strategies have emerged over the past decade to improve outcomes for most lymphoma patients. Nevertheless, the aggressive presentation seen in high-risk mantle cell lymphoma (MCL) patients remains an unmet medical need. The highly proliferative cells that characterize these tumors depend on nucleotide synthesis to ensure high DNA replication and RNA synthesis. To take advantage of this vulnerability, STP-B, a clinically available small molecule selectively targeting CTP synthase 1 (CTPS1) has been recently developed. CTPS1 is a key enzyme of the pyrimidine synthesis pathway mediated through its unique ability to provide enough CTP in highly proliferating cells. Herein, we demonstrated that CTPS1 was expressed in all MCL cells, and that its high expression was associated with unfavorable outcomes for patients treated with chemotherapy. Using aggressive MCL models characterized by blastoid morphology, *TP53* mutation or polyresistance to targeted therapies, we showed that STP-B was highly effective at nanomolar concentrations *in vitro* and *in vivo*, irrespective of these high-risk features. Inhibition of CTPS1 rapidly leads to cell cycle arrest in early S-phase accompanied by inhibition of translation, including of the anti-apoptotic protein MCL1. Consequently, CTPS1 inhibition induced synergistic cell death in combination with the selective BCL2 inhibitor venetoclax, both *in vitro* and *in vivo*. Overall, our study identified CTPS1 as a promising target for MCL patients and provided a mechanism-based combination with the BCL2 inhibitor venetoclax for the design of future chemotherapy-free treatment regimens to overcome resistance.

Introduction

Since the approval of imatinib in the early 2000s, dozens of targeted therapies have been developed and are now part of the therapeutic arsenal for solid and hematological malignancies.[1] Identified as Achilles' heels of mature B-cell lymphomas, the selective inhibition of CD20 (i.e., rituximab, obinutuzumab), BCL2 (i.e., venetoclax) and BTK (i.e., ibrutinib, acalabrutinib) has recently led to remarkable clinical activity. These molecules have shown great promise in combination with chemotherapy,[2,3] and are now being evaluated in combination as first-line chemotherapy-free regimens,[4,5] changing the treatment paradigm for indolent i.e., chronic lymphocytic leukemia (CLL), and aggressive i.e., mantle cell lymphoma (MCL), malignancies. These novel strategies are improving patient survival, but around a third of MCL patients have high-risk disease, which remains associated with rapid relapse and poor outcome.[2,6,7] Biological risk factors characterizing this population, such as high proliferation index (Ki-67), blastoid morphology or *TP53* alterations, are now well described.[8] Nevertheless, aside from the promising initial results obtained with chimeric antigen receptor (CAR) T cells,[9] treatment options for these patients remain an unmet medical need.

Aggressive cancer cells rely on metabolic reprogramming to maintain the hyperactive synthesis of DNA, RNA and phospholipid required to sustain high proliferation and survival.[10] The dependence of MCL cells on high rates of DNA synthesis has been successfully exploited by nucleoside analogs such as cytarabine (ara-C).[11,12] As the treatment of hematological cancers moves away from chemotherapy towards targeted agents, inhibition of nucleotide synthe-

sis remains an attractive strategy, especially by targeting cytidine triphosphate synthases (CTPS). CTPS1 and 2, key enzymes of the pyrimidine synthesis pathway, catalyze the rate-limiting step of CTP *de novo* synthesis.[13,14] Elevated activity of CTPS in cancer was highlighted decades ago, but strategies to target these enzymes have so far resulted in toxicities and limited efficacies *in vivo*.[15,16] A recent surge of interest has come from our better understanding of the differential role of CTPS1 and 2. Indeed, human studies have revealed that individuals carrying an inherited homozygous hypomorphic mutation in *CTPS1* display a marked defect in the proliferation of activated T and B cells, with no phenotype outside of the hematopoietic system.[17,18] These studies suggested that the selective inhibition of CTPS1, but not CTPS2, could be an innovative targeted strategy in aggressive lymphoid malignancies. *In vitro* functional studies further confirmed a specific role of CTPS1 and 2 and *in vitro* loss-of-function experiments (clustered regularly interspaced short palindromic repeats [CRISPR], small hairpin RNA [shRNA]) have demonstrated the dependence of B and T lymphomas, as well as certain solid tumors, on CTPS1 for their proliferation, especially when CTPS2 level is low.[19-23]

STP-B is a highly selective small molecule inhibitor of CTPS1, with more than 1,300-fold selectivity over CTPS2.[24] In the present work, we evaluated the efficacy of pharmacological CTPS1 inhibition as single-agent therapy, and in combination with targeted therapies *in vitro* (cell lines, primary cells) and *in vivo* (cell line, patient-derived xenograft [PDX]), in MCL models displaying high-risk features. Our results demonstrate the efficacy of STP-B in these preclinical models, document its mechanisms of action and highlight a mechanism-based rationale for combination with the BCL2 inhibitor venetoclax.

Methods

Cell culture

Cell lines were authenticated by major histocompatibility complex (MHC) class I sequencing (EFS Nantes, France) and routinely identified using a flow cytometry-based barcode.[25] Samples were collected from peripheral blood (PB) after obtaining informed consent from MCL patients (REFRACT-LYMA cohort; ethical approval GNEDS-2015-09-13). MCL cells were enriched using anti-human CD19-conjugated magnetic beads (Miltenyi®) and cultured using previously described protocols.[26]

Drug testing

Cell lines were treated with a highly selective inhibitor of CTPS1 provided by Step Pharma,[24] STP-B (1-3,000 nM), alone or in combination with venetoclax (0.25-3,000 nM) or ibrutinib (5-500 nM). Viability assays (CellTiter-Glo and Annexin-V staining) were performed 24 to 72 hours (h) after treatment. The synergy score (Bliss) was calculated using 'SynergyFinder' website. Bromodeoxyuridine/propidium iodide (BrdU/PI) cell cycle analysis was performed as previously described.[26]

Transcriptomic analysis

Whole transcriptome sequencing of MCL cell lines cultured or not with STP-B, for 24 h at half maximal inhibitory concentration (IC_{50}), and of MCL cells from patients involved in the REFRACT-LYMA cohort, was performed using the 3'sequencing-RNA Profiling (3'SRP) protocol previously described.[27] Publicly available datasets from bulk and single-cell RNA sequencing (scRNA-seq) of MCL samples were also collected from the Gene Expression Omnibus database (GSE93291, GSE239497, GSE239353) and analyzed as previously described.[28]

In vivo mouse models

Six to 8-week-old NSG mice (N=40, Charles River) were used for the study. The study was conducted within the UTE (Unité Thérapeutique Expérimentale) animal core facility of IRS-UN (C44-278 certification renewed on December 17, 2015). Animal study was approved by Ethics Committee of Pays de la Loire, France (review CEEA.2010.49) and the French Ministry of Higher Education and Research and supported by the animal welfare structure of the UTE animal core facility of IRS-UN. The first and most important criteria throughout the study was the animals' health: any mouse risking to drop 20% of body weight or showing any clinical signs indicating that the animal is not in good health (abnormal behavior, breathing difficulties, coat appearance, etc.) was removed from the study. Venetoclax (InVivoChem) was formulated extemporaneously and protected from light in 5% dimethylsulfoxide (DMSO) / 50% polyethylene glycol300 / 5% Tween80 / 40% ddH_2O. STP-B (provided by Step Pharma) was formulated extemporaneously and protected from light in 10% benzyl alcohol and 90% Castor oil. Z138 and PDX models used in the study are detailed in the *Online Supplementary Appendix*.

Additional methods are detailed in the *Online Supplementary Appendix*.

Results

A high level of CTPS1, but not CTPS2, is predictive of a poor prognosis in mantle cell lymphoma

While the primary expansion zone of lymphomas is located in the lymph nodes (LN), MCL is characterized by early dissemination in virtually all patients, with lymphoma cells circulating in the bone marrow (BM) and peripheral blood (PB).[29] RNA-seq analysis of primary MCL samples showed that *CTPS1* was expressed at significantly higher levels than *CTPS2* in the LN (N=100) as well as in circulating cells (N=72) (Figure 1A, B). A similar expression profile was also

observed in MCL cell lines at the RNA (N=11) and protein (N=6) levels (Figure 1C; *Online Supplementary Figure S1A*). In order to further assess the consequences of CTPS expression on clinical outcomes, we studied *CTPS1/2* gene expression in datasets from MCL patients previously treated with chemotherapy (rituximab, cyclophosphamide, doxorubicin, prednisone, vincristine [R-CHOP], N=122). Patients with high *CTPS1* expression (upper tercile) had significantly poorer overall survival (OS) with median OS of 0.83 year *versus* 4.68 years for the lower expressors (P<0.0001) (Figure 1D). This observation was confirmed in patients treated with an intensive chemotherapy regimen (rituximab, cisplatin, dexamethasone, and high-dose cytarabine [R-DHAP], Lyma-trial,[30] N=98), in which patients with higher *CTPS1* expression had significantly shorter OS (median not reached; P=0.037) (Figure 1E). In contrast, *CTPS2* expression was not significantly associated with OS in either of these cohorts (Figure 1D, E)

Taken together, *CTPS1*, but not *CTPS2*, is expressed in all MCL cells and high levels are associated with unfavorable outcomes, supporting the rationale for its selective targeting in aggressive forms of MCL.

Selective targeting of CTPS1 by STP-B reduces viability of aggressive mantle cell lymphoma cells

Analysis of the DepMap CRISPR dataset[31] suggested that all B-cell lymphoma cell lines were strictly dependent on CTPS1 but not CTPS2 for growth (dependency score mean: -0.93 *vs*. 0.00; N=70) (*Online Supplementary Figure S1B*). Consistent with this, targeting CTPS1 with STP-B, a small molecule inhibitor with high selectivity for CTPS1 over CTPS2,[24] resulted in a dramatic loss of viability in all MCL cell lines tested (N=11, median IC_{50}=220 nM; range, 7-3,100 nM), with all but one cell line having an IC_{50} of less than 1 μM (Table 1; Figure 2A). Response to STP-B was not correlated with *CTPS1/2* mRNA level and was independent of

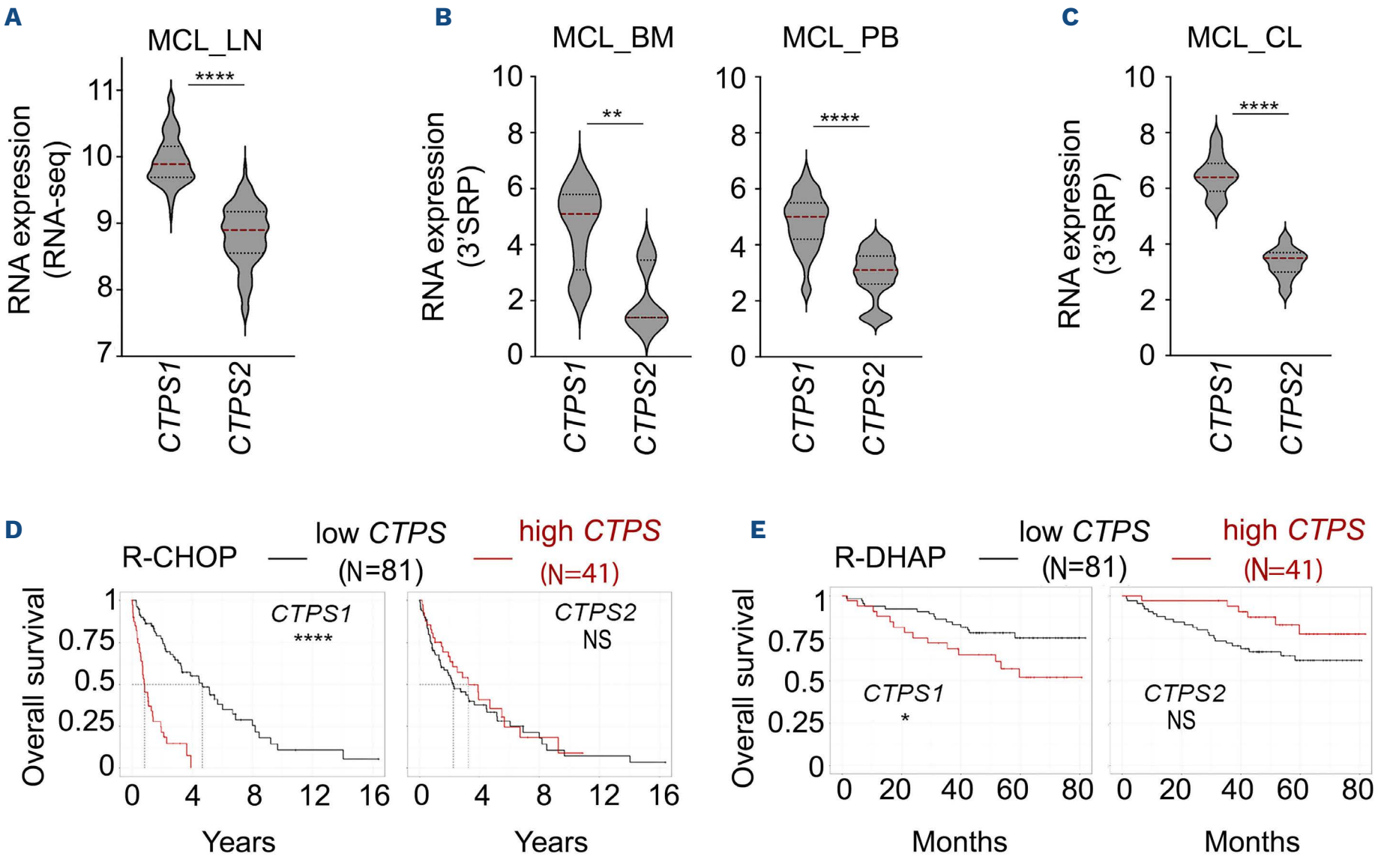

Figure 1. *CTPS1*, but not *CTPS2*, is highly expressed in mantle cell lymphoma and associated with poor prognosis. (A) *CTPS1* and *CTPS2* expression was assessed by RNA sequencing (RNA-seq) in lymph node (LN) biopsies from 100 mantle cell lymphoma (MCL) patients at diagnosis. (B) *CTPS1/2* mRNA levels were analyzed by 3'SRP in MCL cells from bone marrow (BM, N=9) or peripheral blood (PB, N=63) patient samples. (C) *CTPS1/2* expression was determined in 11 MCL cell lines (CL) by 3'SRP. Mann-Whitney test was used. **P<0.01, ****P<0.0001. (D) Overall survival probabilities with different *CTPS1/2* expression were estimated by the Kaplan-Meier method. Probabilities were calculated on 122 MCL patients treated with rituximab, cyclophosphamide, doxorubicin, prednisone, vincristine (R-CHOP) (public dataset GSE93291). ****P<0.0001. (E) Overall survival probabilities with different *CTPS1/2* expression were assessed similarly using data from the LYMA trial (N=98). *P<0.05. NS: not significant; R-DHAP: rituximab, cisplatin, dexamethasone, and high-dose cytarabine; 3'SRP: 3'sequencing-RNA profiling.

high-risk features such as *TP53* mutation and resistance to venetoclax or ibrutinib (Table 1; *Online Supplementary Figure S2A, B*). This observation was corroborated by data from resistant isogenic cell lines: ibrutinib-resistant JeKo-1, venetoclax-resistant Maver-1 and Z138 *TP53*^{KO} (CRISPR/Cas9) cell lines (Figure 2B, C; *Online Supplementary Figure S2C; Online Supplementary Table S1*).

The activity of STP-B as a single agent was confirmed *in vivo* using the ibrutinib/venetoclax double-resistant Z138 xenograft model. As shown in Figure 2D, tumor volume was significantly reduced in STP-B-treated mice compared with control mice, with inhibition of tumor growth after STP-B treatment reaching 46% by day 17. Of note, the 30 mg/kg dose of STP-B used was well tolerated by the mice (*Online Supplementary Figure S3A*). The activity of STP-B as a single agent was confirmed in a disseminated MCL model, using ibrutinib-resistant *TP53*^{MUT} blastoid PDX cells. Over 60% inhibition of circulating MCL cells (huCD45^{+} cells) was achieved after three cycles of treatment (28 days after engraftment) (Figure 2E, left panel), and a significant gain in survival was observed (P<0.01), despite the aggressive phenotype of this model (Figure 2E, right panel). In contrast, the number of mouse peripheral blood mononuclear cells (PBMC) (huCD45- cells) was not significantly altered by treatment (*Online Supplementary Figure S3B*).

Overall, these results demonstrated that MCL cells are CTPS1-dependent and that its selective inhibition by STP-B impairs growth of aggressive MCL *in vivo*.

CTPS1 is preferentially expressed in cycling mantle cell lymphoma cells and its targeting triggers early S-phase arrest

In order to further decipher the consequences of selective CTPS1 inhibition at the molecular level, we performed a transcriptomic analysis in nine MCL cell lines treated or not with STP-B (IC_{50} for 24 h) (*Online Supplementary Figure S4A*). After STP-B treatment, 110 genes showed significantly altered expression (29 up- and 81 downregulated; adjusted P<0.05) (*Online Supplementary Figure S4B*). Functional annotation highlighted the disruption of transcriptional programs associated with translation (translation, cap-dependent translation) and cell cycle (M phase, anaphase, metaphase, cell cycle checkpoints, cell cycle) (Figure 3A). A deeper analysis of cell cycle associated genes highlighted an enrichment of the transcripts expressed in G1_early-S (e.g., *E2F1/2*, *CDK4*, *PIK3IP1* and genes encoding Cyclin D, E and A) and a decrease of late-S_G2/M-associated genes (e.g., *MKI67*, *PLK1*, *UBEC2*, *CDC20* and gene encoding Cyclin B), suggesting that CTPS1 inhibition by STP-B led to S-phase arrest (Figure 3B). Regarding transcription factors, YBX1, MYC and E2F family members (E2F3/4) were predicted to be inhibited by STP-B (TRRUST algorithm), in line with cell cycle modulation and recent studies on CTPS1 regulation.[20,21,23] Using LN gene expression data from two cohorts of MCL patients, we showed that expression of *CTPS1*, but not *CTPS2*, was significantly and positively correlated with proliferation index Ki67 *(MKI67*; P<0.0001) levels (Figure 3C). Such a correlation was also confirmed by scRNA-seq in circulating MCL cells, which are mainly characterized by resting cells and minor cycling subpopulations.[28] Indeed, proliferating BM MCL cells, identified using a previously described cell-cycle signature,[28] expressed higher levels of *CTPS1* compared to resting cells (P<0.0001), while *CTPS2* showed low levels in both populations (Figure 3D). Overall, these transcriptomic data showed that *CTPS1* is highly expressed in proliferating cells and that its selective targeting alters cell cycle transit resulting in arrest in early S-phase.

Functional cell cycle analysis (BrdU/PI staining) of MCL

Table 1. STP-B half maximal inhibitory concentration and mantle cell lymphoma cell line characteristics.

STP-B	IC_{50} values (μM)		RNA-seq (log2)		Characteristics		Other drug responses	
MCL cell lines	Mean	SEM	*CTPS1*	*CTPS2*	EBV	*TP53*	Venetoclax	Ibrutinib
Z138	0.007	0.00	6.3	2.3	-	wt	R	R
SP53	0.019	0.01	6.3	3.5	-	wt	R	S
HBL2	0.032	0.01	6.9	2.7	-	D281G	S	R
JeKo-1	0.036	0.001	5.5	3.0	-	V122*	R	S
UPN1	0.082	0.01	7.8	3.7	-	E286K	R	S
MAVER-1	0.22	0.07	6.6	3.5	-	D281E	S	R
REC-1	0.44	0.10	6.6	3.1	-	G244D	R	S
GRANTA-519	0.52	0.11	7.3	3.2	+	wt	S	R
NTS-3	0.63	0.11	5.4	3.8	-	wt	S	S
JVM2	1.0	0.47	ND	ND	+	wt	R	S
MINO	3.1	0.84	5.9	3.5	-	V147G	S	S

Half maximal inhibitory concentration (IC_{50}) values were determined by Cell-Titer Glo assay in 11 mantle cell lymphoma (MCL) cell lines treated for 72 hours. Values represent the mean of 3 independent experiments. RNA-seq: RNA sequencing; SEM: standard error of the mean; EBV: Epstein-Barr virus; R: resistant (IC_{50} >1,000 nM), S: sensitive (IC_{50} <1,000 nM); wt: wild-type; ND: not determined.

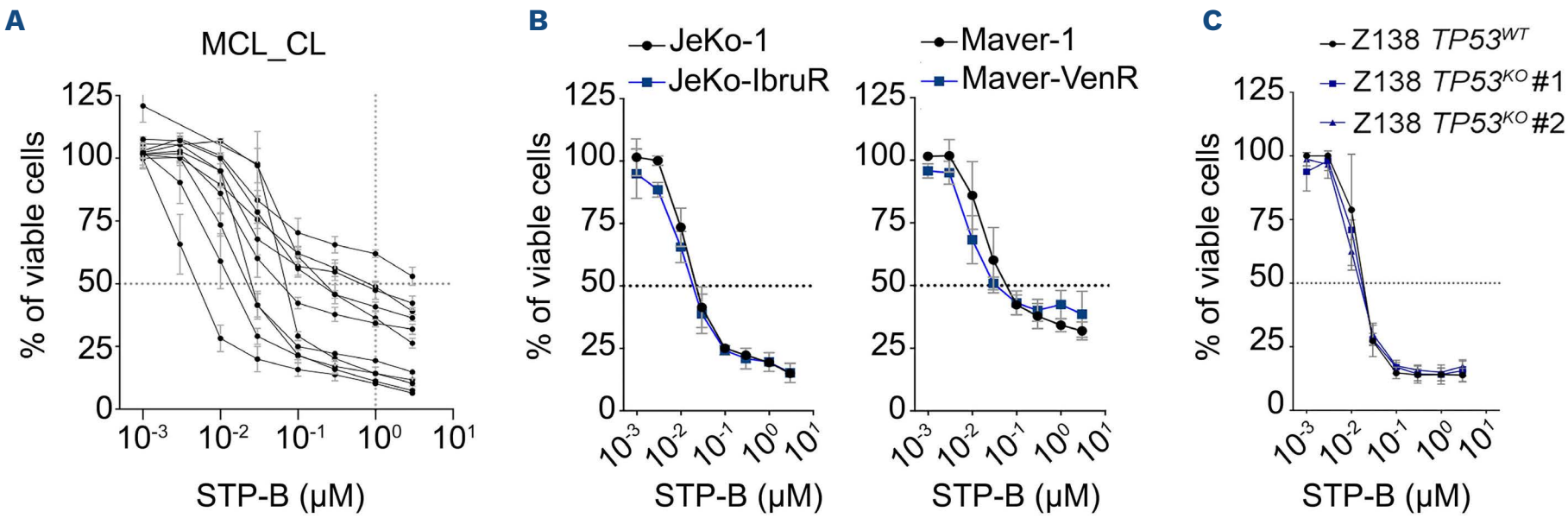

Figure 2. Selective CTPS1 targeting reduces tumor viability in aggressive mantle cell lymphoma preclinical models. (A) Dose response to STP-B was evaluated by CellTiter-Glo (CTG) assay in 11 mantle cell lymphoma (MCL) cell lines treated for 72 hours. (B) Response to STP-B was similarly assessed in JeKo-1 and a derived ibrutinib-resistant JeKo-1 (left graph) and in MAVER-1 and a derived venetoclax-resistant MAVER-1 (right graph). (C) Dose response to STP-B was evaluated by CTG assay in 2 Z138 *TP53*^{KO} clones compared to isogenic Z138 *TP53*^{WT} cells treated for 72 hours. (D) STP-B efficacy was determined *in vivo* using Z138 xenograft model. Mice were treated with vehicle (N=5) or 30 mg/kg STP-B (N=5) 4 consecutive days a week for 3 cycles. Tumor size was measured by caliper. (E) STP-B efficacy *in vivo* was assessed using a disseminated patient-derived xenograft (PDX) model (ibrutinib-resistant, *TP53*^{MUT}, blastoid). Left panels: circulating MCL cells count was determined by flow cytometry (human [hu] CD45^{+}) 21 days and 28 days after engraftment. Mann-Whitney test was used. ***P*<0.01. Right panel: survival of PDX mice treated with vehicle (N=5) or STP-B (N=5) was analyzed. Mantel-Cox and Gehan-Breslow-Wilcoxon tests were performed.

cell lines (N=5) further demonstrated that a 24-h exposure to STP-B resulted in a significant accumulation of early-S phase cells (fold increase 2.0) and a consequent drop in late S and G2/M cells (fold decrease 0.8 and 0.3, respectively) (Figure 4A). This was confirmed in proliferating MCL primary cells (N=11) (Figure 4B). While cell death remained low 24 h after CTPS1 inhibition, a cytotoxic effect was observed from 48 h onwards (lethal dose, 50% [LD_{50}] <120 nM, N=3) (Figure 4C), highlighting that S-phase arrest was followed by massive cell death in highly proliferative cells *in vitro*.

STP-B synergizes with the cytotoxic BCL2 inhibitor venetoclax

In vitro combination studies were then undertaken with two targeted therapies widely used in MCL i.e., ibrutinib, a selective BTK inhibitor, and venetoclax, a selective BCL2 inhibitor. The combination STP-B/venetoclax, but not STP-B/ibrutinib, led to supra-additive apoptosis (mean synergy score: 11.8 and -3.2, respectively, N=9) (Figure 5A; *Online Supplementary Figure S5A, B*). In contrast to the BCL2-negative UPN1 cells (synergy score: 0.3), BCL2-positive Z138, JeKo-1 and MINO cells, as well as their associated ibru-

tinib- and venetoclax-resistant derived cell lines, were synergically killed by the STP-B/venetoclax combination (*Online Supplementary Figure S5B*).

Further experiments demonstrated that pretreatment of the venetoclax-resistant Z138 cells with STP-B resulted in an elevated BCL2 priming at the mitochondrial level (increased cytochrome-C release; $P<0.01$) (Figure 5B). In order to investigate the molecular mechanisms involved

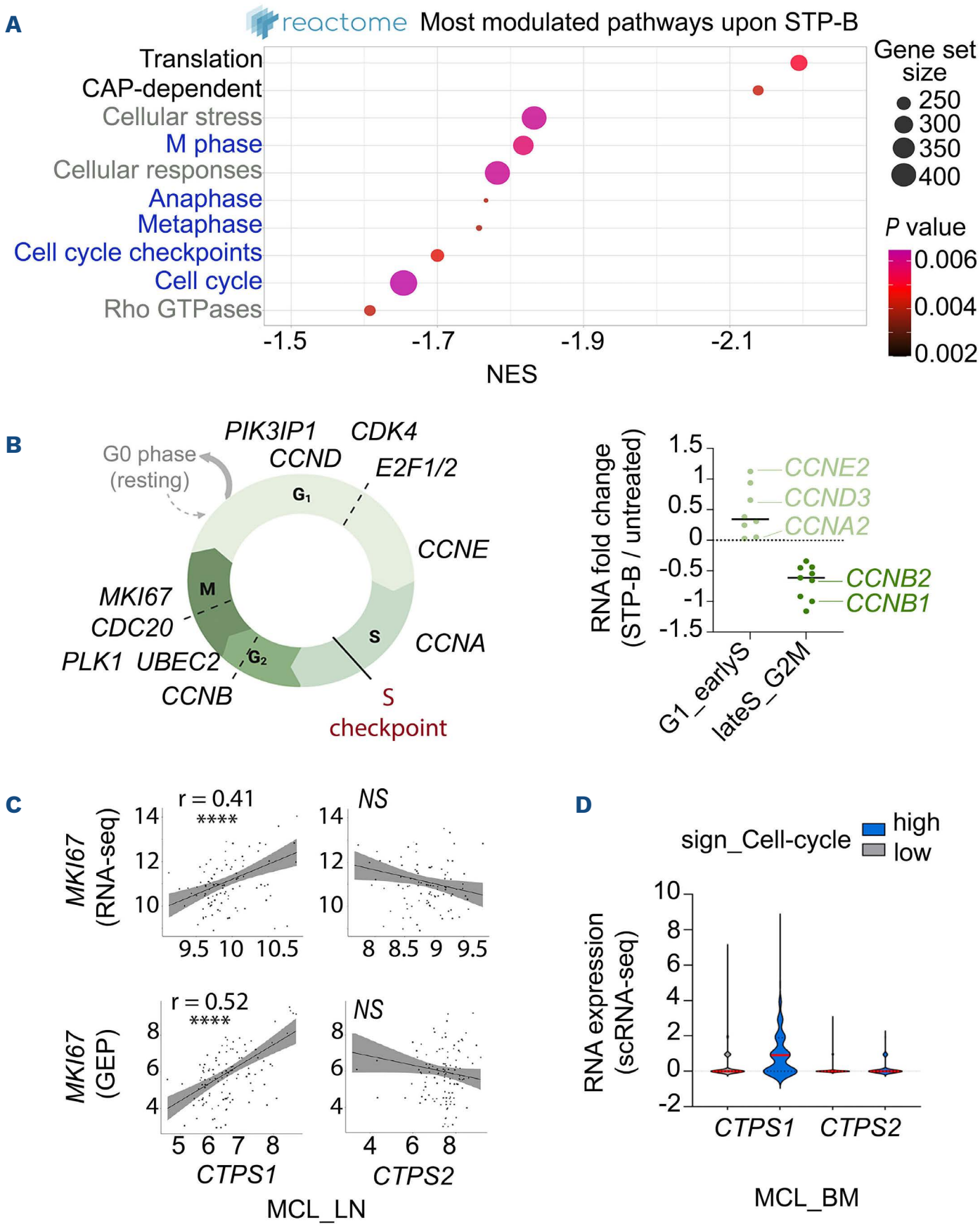

Figure 3. CTPS1 is preferentially expressed in cycling mantle cell lymphoma cells and its inhibition affects cell cycle related transcriptional programs. (A) Nine mantle cell lymphoma (MCL) cell lines were treated with STP-B at half maximal inhibitory concentration (IC_{50}) for 24 hours and genes expression was determined by 3'sequencing-RNA profiling (3'SRP). The top 10 enriched Reactome pathways modulated upon STP-B treatment in MCL cells are depicted. (B) Cell cycle associated genes expression was analyzed upon STP-B treatment. RNA level fold changes (STP-B/untreated) were calculated for genes involved in G1/early-S phases and late-S/G2/M phases. (C) *CTPS1/2* expression was compared to *MKI67* expression in lymph node (LN) biopsies from MCL patients using either RNA sequencing (RNA-seq) data (N=98, upper panels) or gene expression profiling data (N=122, lower panels). Spearman test was used. (D) *CTPS1* and *CTPS2* levels were assessed by single-cell RNA-seq in highly proliferating and resting cells from 6 bone marrow (BM) samples from MCL patients (red line: median).

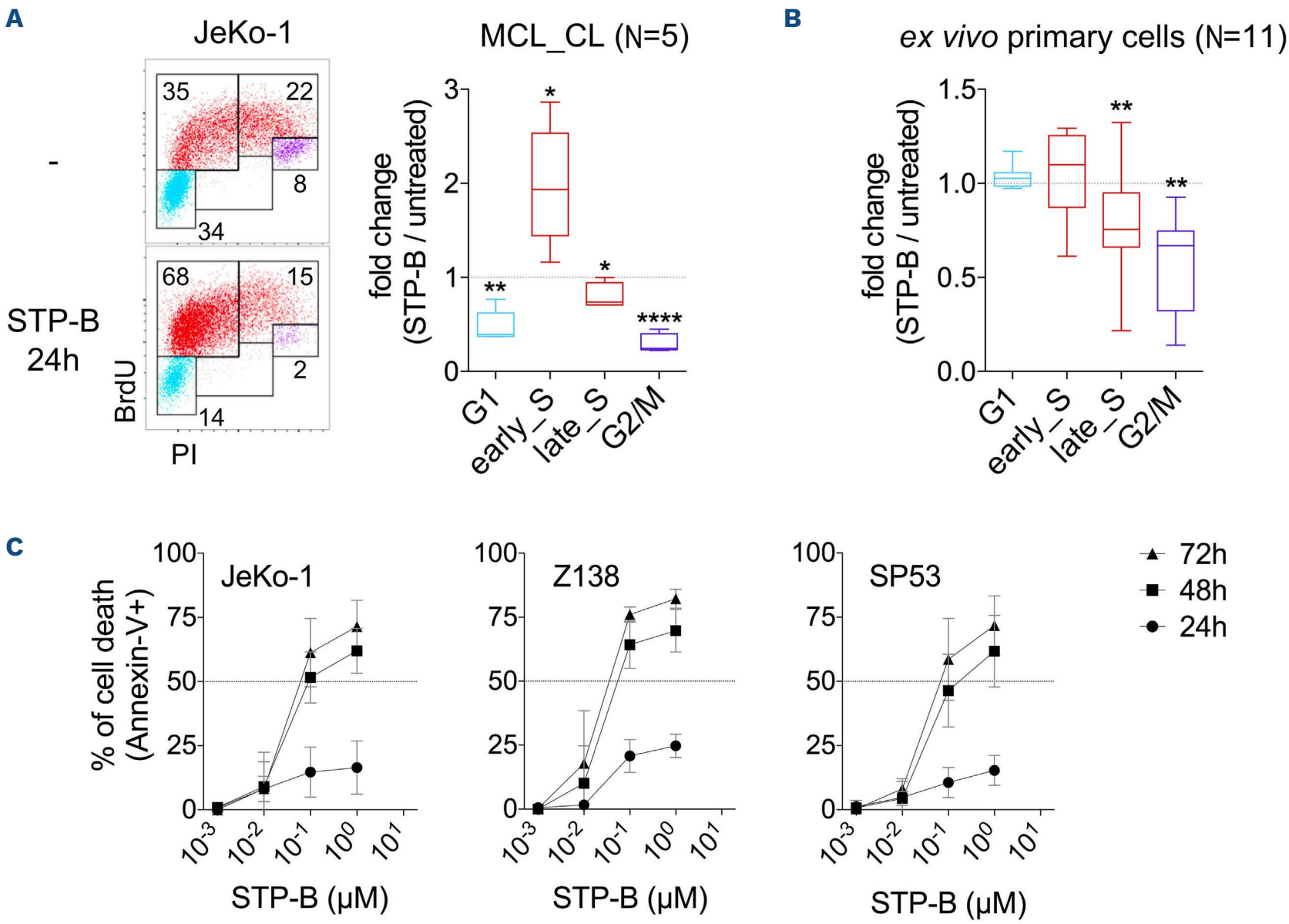

Figure 4. CTPS1 inhibition results in rapid early S-phase arrest followed by late cell death in mantle cell lymphoma. (A) Cell cycle analysis (bromodeoxyuridine/propidium iodide [BrdU/PI]) was performed in 5 mantle cell lymphoma (MCL) cell lines treated for 24 hours (h) with STP-B at half maximal inhibitory concentration (IC_{50}). Percentage of cells in G1, early-S, late-S and G2/M phases is indicated. Change in cell cycle distribution was calculated. Paired *t* test was used. **P*<0.05, ***P*<0.01, *****P*<0.0001. (B) BrdU/PI analysis was performed in 11 MCL primary samples. Primary cells were co-cultured for 72 hours (h) with L40 cells and cytokines to mimic tumor microenvironment and stimulate cell proliferation prior to STP-B treatment (100 nM) for 24 h. Change in cell cycle distribution was calculated. Paired *t* test was used. ***P*<0.01. (C) Cytotoxic activity of STP-B was evaluated at 24, 48 and 72 h by Annexin-V staining in 3 MCL cell lines. Graphs represent 4 independent experiments.

in the observed synergy, we first studied the regulation of the anti-apoptotic proteins MCL1 and BCL-XL, two major venetoclax resistance factors located at the mitochondria,[32] in Z138 and JeKo-1 cells treated with STP-B for 24 h. While BCL2 and BCL-XL protein levels remained unchanged, STP-B treatment resulted in a rapid degradation of MCL1 protein in both cell lines, even at low STP-B concentrations (Figure 5C). Quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that *MCL1* mRNA levels did not significantly change after STP-B treatment, suggesting that a defect in the translational machinery is involved in MCL1 depletion (*Online Supplementary Figure S6A*). Post-transcriptional inhibition of additional proteins translated from so called weak mRNA,[33,34] such as *CCND1*, *CCND2* and *BIRC5* (*Online Supplementary Figure S6B, C*), as well as the enrichment of transcriptomic functional annotations related to translation (Figure 3A), supported such a mechanism for MCL1 inhibition by STP-B. Decreased phosphorylation of the cap-dependent translation inhibitor 4E-BP1 upon STP-B treatment further suggested that translation was impaired (*Online Supplementary Figure S6D*). Finally, puromycin incorporation assay confirmed a reduced protein synthesis upon STP-B treatment in both Z138 and JeKo-1 cell lines (Figure 5D). Overall, these results confirmed that CTPS1 inhibition impaired translation, leading to decreased MCL1 protein levels.

Finally, to address CTPS1/BCL2 dual targeting in aggressive MCL cells *in vivo*, we used the venetoclax-resistant Z138 xenograft model. While treatment with venetoclax resulted in limited inhibition of tumor growth, STP-B reduced tumor growth to 71% of control at day 17. The combination was more effective, with tumor inhibition reaching 87% compared to control at day 17. Importantly, the synergy was also observed in the latter stages, with tumor mass in mice treated by STP-B alone increasing 2.3-fold between day 17 to day 22, whereas mice treated by the STP-B/venetoclax

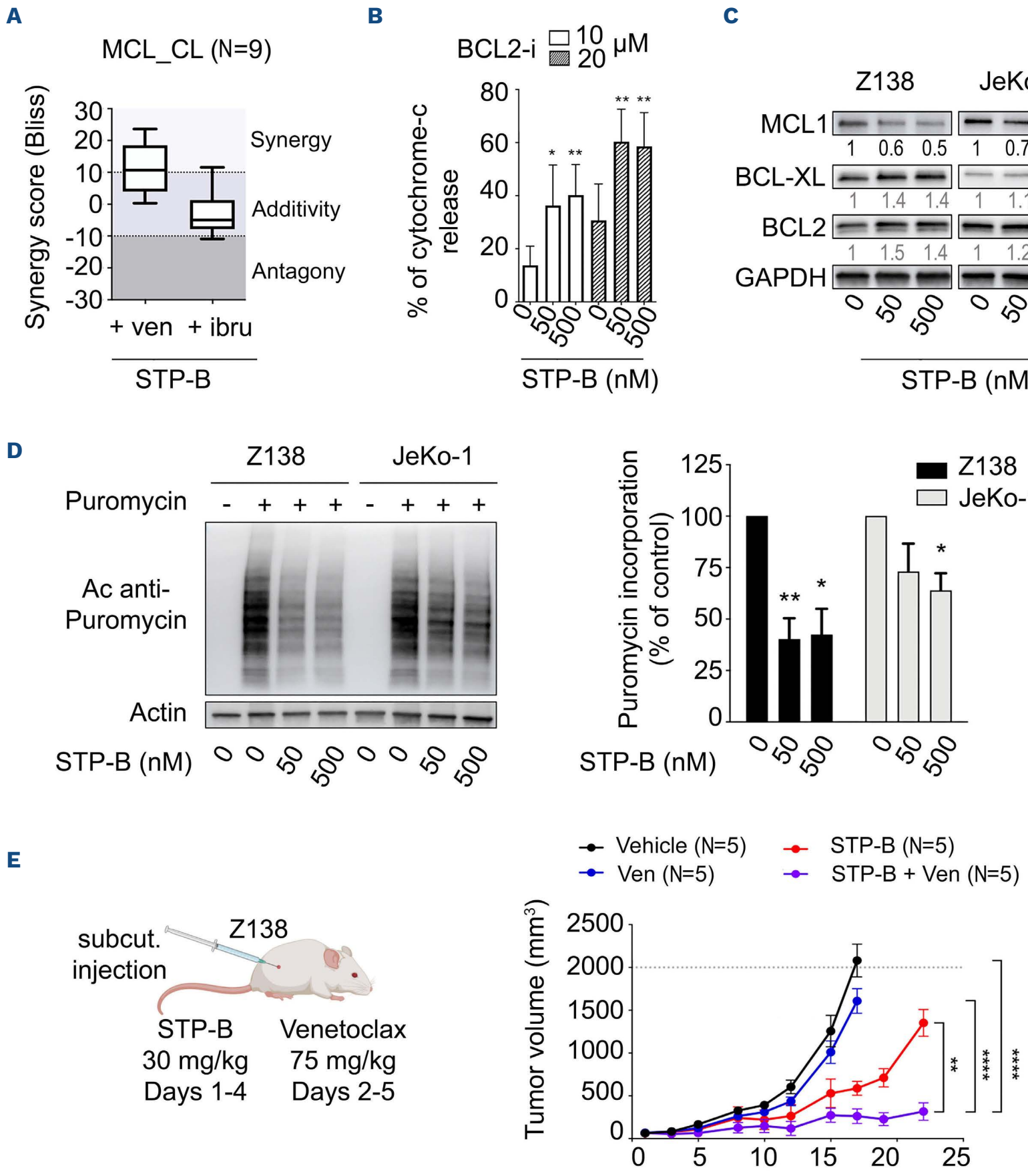

Figure 5. CTPS1 inhibition synergizes with BCL2 inhibition *in vitro* and *in vivo*. (A) Bliss synergy scores were determined after treatment for 72 hours (h) with STP-B/venetoclax (ven) or STP-B/ibrutinib (ibru) combinations in mantle cell lymphoma (MCL) cell lines. Detailed results are shown in *Online Supplementary Figure S5*. (B) BCL2 dependence following STP-B treatment was assessed by BH3 profiling in Z138. Cells were pretreated for 24 h with 50 or 500 nM STP-B. Cytochrome C release was analyzed after treatment with 10 or 20 µM of ven (BCL2-i) as indicated in the *Online Supplementary Appendix*. Graph represents 4 independent experiments. Paired *t* test was used. **P*<0.05, ***P*<0.01. (C) Immunoblotting of anti-apoptotic BCL2 family members was performed in Z138 and JeKo-1 treated with STP-B at 50 and 500 nM for 24 h. Protein levels normalized to GAPDH level are indicated. (D) Puromycin incorporation assay was performed to directly evaluate the rate of protein synthesis upon STP-B treatment. Cells were pretreated for 24 h with 50 or 500 nM STP-B, prior to puromycin addition as indicated in the *Online Supplementary Appendix*. Graph indicates the percentage of puromycin incorporated. (E) The efficacy of STP-B/ven combination was evaluated *in vivo* using a Z138 xenograft model (N=5 mice per group). STP-B was dosed at 30 mg/kg days 1-4 of a 7-day cycle and ven was dosed at 75 mg/kg days 2-5 of a 7-day cycle for 3 cycles. Statistical analysis was made using a two-way ANOVA test followed by a Tukey's multiple comparisons test. ***P*<0.01, *****P*<0.0001. subcut.: subcutaneous.

combination showed stable disease after all three cycles of treatment (1.2-fold increase from day 17 to day 22) (Figure 5E). Both single and combined compounds were well tolerated, with the mean variation in body weight in all groups being less than 10% (*data not shown*).

Discussion

Despite increasing treatment options, around a third of MCL patients are refractory to chemotherapy and respond poorly to targeted therapies.[35] These high-risk patients are now better characterized, but innovative treatment strategies are still limited.[8] Here we show that targeting nucleotide metabolism by selective inhibition of CTPS1, using the small molecule STP-B, effectively reduces viability in preclinical models of highly aggressive MCL *in vitro* and *in vivo*. STP-B has shown efficacy at nanomolar levels *in vitro*, and in mouse models at concentrations achievable in human patients,[24] irrespective of high-risk features such as *TP53* gene deletion or mutation, high proliferation index (Ki-67) or blastoid morphology.

In this study, based on transcriptomic analysis and functional validations, we demonstrated that targeting the pyrimidine synthesis pathway in MCL with STP-B resulted in rapid cell cycle arrest. All MCL cases are characterized by a deregulated cell cycle resulting from Cyclin D overexpression, mainly due to a chromosomal translocation t(11;14)(q13;q32),[36] and additional hits in the cell cycle such as *CDKN2A* deletion are associated with resistance to chemotherapy.[37] This unrestrained proliferation suggested that targeting the cell cycle could be a sustainable strategy, particularly in aggressive MCL, which has been confirmed by the clinical efficacy of CDK4/6 inhibitors.[38,39] Nevertheless, as with many targeted therapies, cancer cells have shown multiple ways of escape, notably through RB1 inactivation, which leads to CDK4/6-independent proliferation.[40,41] In contrast, CTPS1, catalyzing a rate-limiting step in CTP synthesis, is essential for lymphoma growth, as recently demonstrated by gene editing.[19,21] Accordingly, *CTPS1* expression was detected in all MCL samples tested (RNA-seq: N=182), suggesting that *CTPS1* loss is infrequent. In addition, *CTPS1* expression was higher in samples with high proliferation (high *MKI67*) and was also described as higher in ibrutinib-refractory patients, who are characterized by a poor prognosis.[7,42] Taken together, CTPS1 appears to be an appealing target to impair the cell cycle of high-risk lymphomas.

In contrast to *CTPS1*, *CTPS2* was expressed at low levels in MCL, did not correlate with *MKI67* and was not associated with a poor prognosis. Despite sharing 74% homology at the protein level, CTPS1 and CTPS2 have differential roles in cell proliferation. Indeed, a recent study suggests that CTPS2 has weaker enzymatic activities and may be involved in maintaining a basal cellular CTP level, and cannot compensate for the role of CTPS1 in high proliferation.[19] This is consistent with human studies showing that CTPS1 homozygous mutations resulted in the inability of activated lymphocytes to highly proliferate, even in the presence of normal CTPS2 levels.[17,18] On the basis of these data, CTPS2 is unlikely to compensate for the loss of CTPS1 enzymatic activity in tumors treated with STP-B. Nevertheless, given the high plasticity of tumor cells under long-term drug pressure, its level should be carefully monitored in future clinical samples if a CTPS1-targeting refractory patient population emerges. A compound from this chemical series entered clinical development for relapsed refractory B and T lymphomas in 2022 (*clinicaltrials gov. Identifier: NCT05463263*).

Since CTPS1 inhibition preferentially targets proliferating MCL cells, we next tested the efficacy of the combination with venetoclax, a cell-cycle-independent cytotoxic agent. Venetoclax is a BH3-mimetic that selectively targets BCL2 at the mitochondria and induces rapid apoptosis in lymphoma cells such as MCL cells. BCL2 inhibition has shown good clinical activity as a single agent, but tumor cells have the ability to rapidly develop resistance, especially through the upregulation of other anti-apoptotic proteins such as MCL1.[43] Accordingly, simultaneous inhibition of BCL2 and MCL1, using specific BH3-mimetics, enabled highly effective tumor control in MCL PDX.[44] Unfortunately, direct inhibition of MCL1 by selective BH3 mimetics resulted in unexpected cardiac toxicity[45] and alternative strategies to indirectly neutralize MCL1 are being investigated.[46,47] Here, we demonstrated that inhibition of CTPS1, by altering translation, resulted in rapid inhibition of MCL1, but not BCL2, in MCL cells. MCL1 inhibition led to STP-B/venetoclax synergy *in vitro*, as well as effective tumor control in aggressive mouse models of MCL. These results pave the way for the study of such a combination in the near future, particularly for high-risk MCL cases which currently have very few effective therapeutic options.

De novo pyrimidine synthesis is controlled upstream by mTORC1/S6K1 through CAD phosphorylation.[48] Here we show that selective downstream targeting of CTPS1 results in inhibition of protein synthesis, at least partly via a decrease in mTORC1 activity (inhibition of 4E-BP1 phosphorylation) (*Online Supplementary Figure S6D*). These results highlight the complexity of feedback regulation occurring in the mTORC1 pathway, which needs further mechanistic studies to potentially uncover new vulnerabilities in lymphomas.

Disclosures

HA, AP and PB are employees of Step Pharma. All other authors have no conflicts of interest to disclose.

Contributions

RD designed and performed the experiments, analyzed data and participated in writing the article. CB performed the experiments and bioinformatics analysis and analyzed

data. CD, EM and CK performed experiments and analyzed data. CK and RD performed experiments on NSG mice and analyzed data. BT participated in the design of the study. HA, AP, PB and CPD participated in the design of the study, in the data analysis, and in writing the article. DC designed the study, analyzed data, and wrote the article.

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Data-sharing statement

RNA-sequencing datasets are publicly available in the Gene Expression Omnibus. All other datasets analyzed during the current study are available from the corresponding author on reasonable request.

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