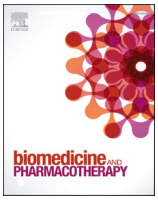
Biomedicine & Pharmacotherapy 190 (2025) 118398

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



Inhibition of NEDDylation enhances the cytostatic effect of Rohinitib on chronic lymphocytic leukemia cells

Jose Luis Castaño [a,1], Víctor Arenas [a,2], Juan José Domínguez [b,3], Sara Fernández-Luis [b,4], Lucrecia Yáñez [b,5], Carlos Pipaón [a,*,6]

[a] Laboratorio de Hematología Molecular, Instituto de Investigación Marqués de Valdecilla Santander, Spain
[b] Servicio de Hematología, Hospital Universitario Marqués de Valdecilla, Spain

ARTICLE INFO

Keywords:
Chronic lymphocytic leukemia
MLN4924
Pevonedistat
Rocaglates
NEDD8
RNA translation

ABSTRACT

Chronic lymphocytic leukemia (CLL) is an incurable hematologic neoplasm that primarily affects elderly individuals. Its treatment consumes a significant amount of clinical resources, a demand expected to grow due to increasing life expectancy in our society. Two characteristics of B-CLL lymphocytes that have been scarcely explored as therapeutic targets are their high RNA translation and increased ubiquitin-like post-translational modifications (UBL-PTM). Previous studies have analyzed the effects of inhibiting these two processes in B-CLL cells separately, yielding promising results. Here we present data demonstrating that the combined inhibition of both processes using the compounds Rohinitib and MLN4924/Pevonedistat synergistically enhances the individual effects of each through the induction of apoptosis. This effect appears to be specific to CLL, as it is not significantly relevant in healthy lymphocytes or in other prevalent hematologic neoplasms, such as multiple myeloma. A molecular characterization of this synergistic effect was conducted, revealing RNA translation dysregulation of NAE1 and UBE2M, as well as kinases involved in BCR signaling, in CLL tumor cells. Additionally, a cooperative regulation of BCL2 and TP53 expression was observed. These preclinical observations support future studies for their therapeutical application in CLL.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a hitherto incurable hematologic neoplasm characterized by the accumulation of mature-looking B lymphocytes that have an extended lifespan in peripheral blood but ultimately invade the bone marrow and lymphoid organs, altering their normal function [1]. CLL primarily affects elderly individuals in Western countries, with a higher incidence in men than in women. As life expectancy improves in our societies, a worsening in the incidence of CLL is expected. Genomic efforts to molecularly characterize CLL have unveiled an extensive but low-prevalence mutational spectrum spanning eight different signaling pathways [2]. Recently, overexpression of wild type RRAS2 has been proposed as a driving molecular event in this neoplasm [3]. Proteomic observations have further expanded its molecular landscape, reporting alterations in post-translational modifications and a translational dependence in the activation of oncogenes.

Dysregulation of mRNA translation characterizes many types of tumors [4]. The rate-limiting step in mRNA translation is the recruitment of 40S ribosomes to the 5'-cap of mRNAs, which is regulated by the heterotetrameric eukaryotic initiation factor complex eIF4F, composed of eIF4A, eIF4B, eIF4G and eIF4E. Thus, eIF4F has become the pharmacological target in current efforts to block oncogene mRNA translation [5,6]. In CLL, elevated expression of oncogenes like c-Myc or Mcl-1 has been linked to the overexpression of the eukaryotic

* Correspondence to: Laboratorio de Hematología Molecular (HEMMOL), Hospital Universitario Marqués de Valdecilla, Torre C, planta -1, 39008 Santander, SPAIN.
E-mail address: carlos.pipaon@scsalud.es (C. Pipaón).
[1] ORCID JLC: 0000–0001-7670–5515
[2] ORCID VA: 0000–0003-4548–6422
[3] ORCID JJD: 0000–0001-6210–1294
[4] ORCID SFL: 0000–0001-9630–5893
[5] ORCID LY: 0000–0001-9925–3048
[6] ORCID CP: 0000–0002-3851–6710

https://doi.org/10.1016/j.biopha.2025.118398
Received 2 May 2025; Received in revised form 18 July 2025; Accepted 29 July 2025
Available online 4 August 2025

translation initiation factor eIF4A [7]. The eIF4A family consists of three closely related proteins: eIF4A1, eIF4A2 and eIF4A3. eIF4A1 is a helicase, involved in resolving secondary structures in the 3'-untranslated regions of certain mRNAs, thereby favoring their translation at the ribosome [8]. Indeed, some research groups are investigating the targeting of eIF4A activity as a potential therapy for CLL [9].

As previously mentioned, growing evidence support a role of ubiquitin-like post-translational modifications (UBL-PTM) in the pathophysiology of CLL. Among them, NEDDylation is receiving special attention, as an inhibitor of the NEDD8 activating protein NAE1, MLN4924/Pevonedistat, has shown cytotoxic activity against B-CLL cells ex vivo [10,11]. Our group has demonstrated elevated NEDDylation in CLL, affecting most of the signaling pathways involved in its development [12,13]. Although MLN4924 has demonstrated therapeutic activity against several neoplasms, its mechanism of action and potential as combinational agent with standard-of-use therapies are still not fully explored [14].

In the present work we report a synergistic cooperation between MLN4924/Pevonedistat and the eIF4A1 inhibitor Rohinitib in the induction of cell death of B lymphocytes from CLL patients. Most importantly, this cytostatic effect is much lower in B lymphocytes obtained from healthy donors. When exploring the molecular bases of this action, we unveiled a previously unreported translational control of kinases involved in BCR signaling.

2. Materials and methods

2.1. Patient samples

Surpluses of peripheral blood samples from CLL patients were used after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation in a Ficoll (GE Healthcare, Uppsala, Sweden) gradient. Characteristics of the patients are detailed in supplementary material.

2.2. Flow cytometry

PBMNC from CLL patients were treated ex-vivo with 250 nM MLN4924, 25 nM TAK243 (Cayman Chemical, Ann Arbor, Michigan, USA) and/or 50 nM Rohinitib (MedChemExpress, Sollentuna, Sweden) as indicated, and cell death was measured by flow cytometry after staining with 7-aminoactinomycin D (7-AAD) in a DxFLEX Flow Cytometer (Beckman Coulter, Indianapolis, USA) and with fluorescent labeled antibodies against CD19 and CD5 to discriminate tumoral B cells. Cell cycle of these cells was also analyzed by flow cytometry after staining with propidium iodide. The combinational treatment of CLL with Rohinitib and Pevonedistat is patent pending.

2.3. Cell viability assay and synergy quantification

250000/well B-CLL cells were plated on 96-well plates and incubated for 48 h in the presence of the indicated drugs in triplicates. Cell viability was then determined using the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] Cell Proliferation kit II (Biotium, Fremont, USA) following the manufacturer's instructions. The synergy degree between the different compounds was calculated using the web tool SyneryFinder [15].

2.4. Screening of the ubiquitin-like post-translational modifications (di-Gly screening)

To analyze the global UBL-PTM profile in CLL we used 2 × 10^8 B cells derived from CLL patients or from buffy coats of healthy donors separated using a CD19 immunomagnetic procedure (Miltenyi Biotec, Bergisch Gladbach, Alemania). Cells were treated with 250 nM MLN4924 (Cayman Chemical, Ann Arbor, Michigan, USA) or an equivalent volumen of DMSO and the level of UBL-PTMs was determined using the UbiScan PTM platform (Cell Signaling Technology, Danvers, EEUU).

2.5. Western blot

Cells were washed with PBS and whole cell lysates were obtained by lysing them in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, USA). Protein concentration was determined by BCA following the manufacturer's instructions (G Biosciences, St. Louis, USA). Proteins (25 μg) were resolved by SDS-PAGE and transferred to PVDF filters (Cytiva, Little Chalfont Buckinghamshire, UK). Blots were incubated with antibodies against NEDD8 (Y297) (AbCam, Cambridge, UK), eIF4A1 (H-5), PDCD4 (B-4), LYN (H-6), SYK (4D10), BTK (7F12H4), PLCɣ2 (B-10), NAE1 (C-2), UBE2M (D-4), GAPDH (0411) (Santa Cruz Biotechnology, Santa Cruz, USA) and then incubated with an IgG kappa-binding protein or an anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, USA). Bound protein was detected by a chemiluminescence assay (Cytiva, Little Chalfont Buckinghamshire, UK) in a LAS4000 mini imager (GE Healthcare, Little Chalfont Buckinghamshire, UK).

2.6. RT-qPCR

Total RNA was prepared using the NZY Total RNA Isolation kit (NZYtech, Lisboa, Portugal). To assess mRNA expression, a reverse transcriptase–SYBR green quantitative polymerase chain reaction (RT-qPCR) method was used. For the RT reaction, RNA (1 μg) was primed with random hexamers and reverse transcribed with Superscript MMLV reverse transcriptase (Invitrogen, Carlsbad, USA) in a 20 μl volume following the manufacturer's instructions. The generated cDNA was analyzed for the expression levels of different genes by real time SYBR green PCR in an Applied Biosystems 7300 machine. The primers used for the analyzed genes are specified at the supplementary material. The measurements were made in triplicates and normalized against those of ß-actin as housekeeping gene.

2.7. Global RNA synthesis assay

For the analysis of nascent protein synthesis in cellular extracts we used the Clik-iT Plus OPP protein synthesis assay kit (Life technologies, Carlsbad, USA) following the manufacturer's instructions. Cell staining was measured by flow cytometry in a DxFLEX Flow Cytometer (Beckman Coulter, Indianapolis, USA). The fluorescence value obtained from cells treated with 30 μM cycloheximide for 30 min was used as the baseline.

2.8. Transcriptome analysis

PBMNC from 5 CLL patients were treated ex vivo with 250 nM MLN4924 (Cayman Chemical, Ann Arbor, Michigan, USA) and/or 50 nM Rohinitib (MedChemExpress, Sollentuna, Sweden) or an equivalent volume of DMSO for 16 h. Total RNA was extracted with the NZY Total RNA Isolation kit (NZYtech, Lisboa, Portugal) and sent to Eurofins Genomics (Ebersberg, Germany), for its analysis. Low expressed genes were removed with CPM value less than 1. The abundance counts of each gene were then used to perform differential gene expression (DGE). DGE was performed using R/Bioconductor package edgeR package. The calcNormFactors function normalizes for RNA composition by finding a set of scaling factors for the library sizes that minimize the log-fold changes between the samples for most genes. Statistical tests were performed for each gene to compare the distributions between conditions (treatment vs control) generating p-values for each gene. The final p-values were corrected by determining false discovery rates (FDR) using the Benjamin–Hochberg method. Using a FDR corrected p-value (adjusted p-value).

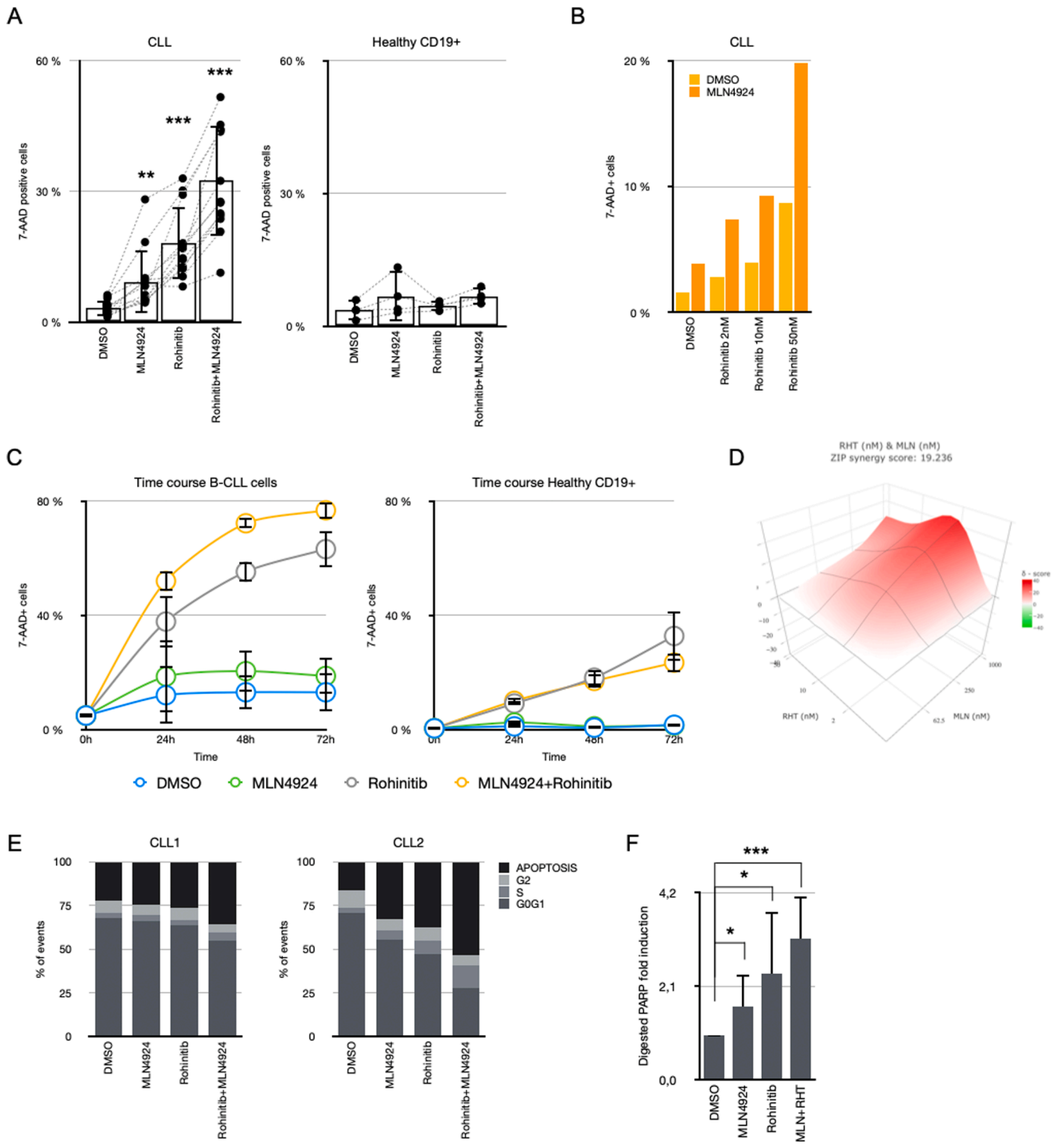


Fig. 1. MLN4924 potentiates B-CLL cell death induced by Rohinitib. A. Flow cytometry analysis of the percentage of 7-AAD-stained B lymphocytes from CLL patients (left panel, n = 15) or healthy donors (right panel, n = 3 pools of 4 donors each), after treatment with 250 nM MLN4924, 50 nM Rohinitib or the combination of both for 24 h. Statistical significance relative to DMSO treated was determined using Student́s T-test (** $p < 0.01$, *** $p < 0.001$). B. A representative Rohinitib dose-response study in the presence or absence of 250 nM MLN4924 for 24 h over B cells from a CLL patient. C. Time course analysis of cell death induction by 250 nM MLN4924 and/or 50 nM Rohinitib over B cells from CLL patients (n = 3) or healthy donors (3 pools of 5 donors each). D. Rohinitib and MLN4924 synergy study over B-CLL cell viability by a tetrazolium salts (XTT) assay at 48 h. Data were analyzed using the SynergyFinder platform. Representative analysis in one patient is shown. E. Cell cycle analysis of B lymphocytes from two CLL patients treated with 250 nM MLN4924, 50 nM Rohinitib or a combination of both for 24 h. F. PARP digestion analysis in B-CLL samples treated with 250 nM MLN4924 and/or 50 nM Rohinitib for 16 h. Densitometric analyses of the digested PARP bands from 6 different CLL samples relative to GAPDH housekeeping gene were performed and the mean fold variations and standard deviations are depicted. Statistical significance relative to DMSO treated was determined using Student́s T-test (* $p < 0.05$, *** $p < 0.001$).

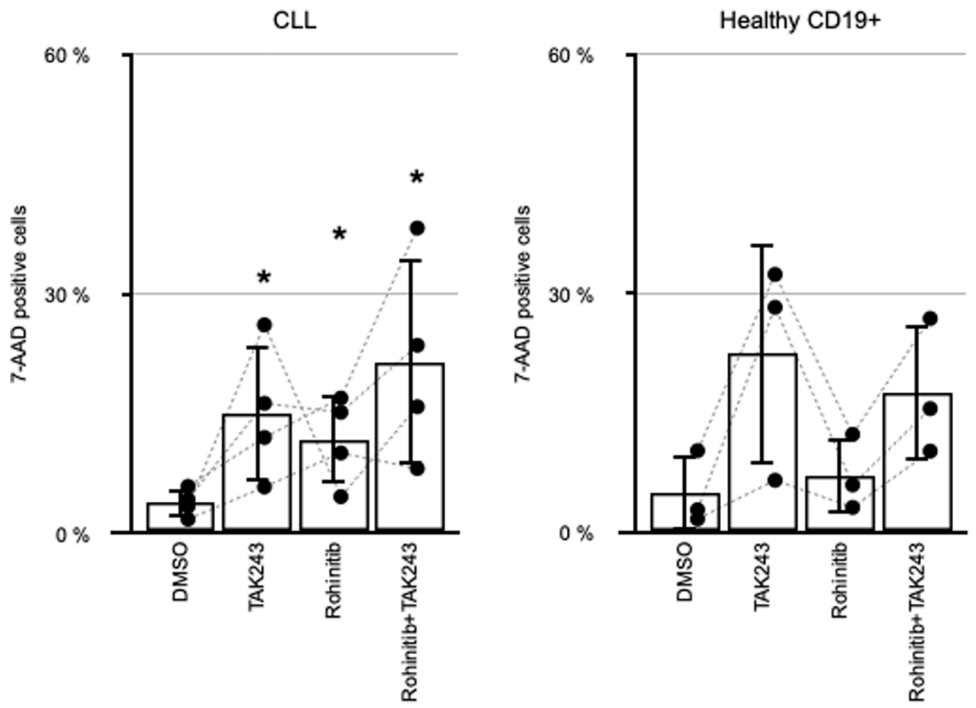


Fig. 2. TAK243 (MLN7243) exerts a milder potentiation than MLN4924 of B-CLL cell death induced by Rohinitib. Flow cytometry analysis of the percentage of B lymphocytes from CLL patients (left panel), or healthy donors (right panel) stained with 7-AAD after 25 nM TAK243, 50 nM Rohinitib or the combination of both for 24 h. Statistical significance relative to DMSO treated was determined using Student́s T-test (* $p < 0.05$).

3. Results

3.1. MLN4924 synergizes with Rohinitib in B-CLL cell death induction treated ex-vivo

We investigated the effect of the combined inhibition of NAE1 and eIF4A1 on the viability of peripheral blood B cells from a cohort of patients, treated ex vivo. As seen in Fig. 1A, both Pevonedistat and Rohinitib alone induced a significant increment in B-CLL cell death. However, the combinational treatment with both drugs potentiated their effect. Interestingly, cell death induction by these treatments was much lower in B lymphocytes obtained from healthy donors (Fig. 1A). The induction of cell death was dose- and time-dependent (Fig. 1B-C and supplementary figure 1) and exhibited an elevated degree of synergism (Fig. 1D). The synergy between MLN4924 and Rohinitib was not observed in cell lines from another prevalent hematologic neoplasm, multiple myeloma (supplementary figure 2). The B-CLL cell cycle analysis under the different treatments showed an increment in the number of events in the sub-G0 peak, suggesting the activation of the apoptosis machinery (Fig. 1E). Supporting this activation, a significant increase in PARP digestion was observed in these cells (Fig. 1F). We analyzed how three prognosis factors like gender, age at diagnosis and the mutational status of the variable region of the immunoglobulin heavy chain (IgHV) gene, related to the response to the combinational treatment. We did not find differences in the response among these groups, although the number of cases analyzed to this end was low (supplementary figure 3).

Since NEDDylation modulates the polyubiquitination mediated by Cullin-RING ligases (CRL), we investigated whether the effects of MLN4924 could also be obtained using an inhibitor of ubiquitin-activating enzyme UAE, TAK243. We observed that TAK243 induces cell death of either CLL and healthy B lymphocytes at 10 times less concentration than MLN4924 in monotherapy. However, the potentiation of Rohinitib action was much milder (Fig. 2 and supplementary figure 4). These results demonstrate that the alteration in protein translation and UBL-PTM are characteristics of CLL that collaborate in B-CLL cell survival and can be used as therapeutic targets.

3.2. BCR signaling kinases exhibit higher mRNA translation levels in CLL

In line with previously published data, we observed an elevated accumulation of eIF4A1 protein in B-CLL lymphocytes, compared to those obtained from healthy donors. Similarly, we also found an accumulation of PDCD4 protein, the natural inhibitor of eIF4A1 (Fig. 3A).

Given that BCR signaling is another common characteristic of B-CLL cells and a primary target in current therapies, we were interested in exploring the potential relationship between these two processes. Stimulation of BCR receptor has been related to an increment of global mRNA translation along with the expression of eIF4A and eIF4G1 [16]. We investigated whether the expression of proteins involved in BCR signaling, like BTK, SYK, LYN or PLCɣ2, were reciprocally influenced by the increment in mRNA translation regulation in B-CLL cells. As presented in Fig. 3B, treatment of B-CLL cells with Rohinitib reverted the elevated accumulation of LYN, SYK, BTK and PLCɣ2. This decline in protein accumulation was accompanied by an accumulation of the mRNAs of LYN, BTK and PLCG2, similar to what happens with other mRNA translation-regulated genes (Fig. 4) [7]. These results suggest that the elevated protein expression of BCR signaling kinases observed in B-CLL lymphocytes are at least in part due to an increment in their mRNA translation.

3.3. eIF4A1 inhibition reverts the overexpression of some NEDDylation cascade proteins

We wondered about the molecular connection between mRNA translation control and UBL-PTM. We started by investigating the putative influence of protein translation dysregulation over the increment in NEDDylation in CLL. We observed how the inhibition of mRNA translation with Rohinitib induced a reduction in the NEDDylation of Cullins (Fig. 5A). Concurrently, Rohinitib induced a decline in UBE2M and NAE1 protein accumulation and an increment of their mRNAs, just like other translation-regulated genes were described to respond [7] (Fig. 5B-C).

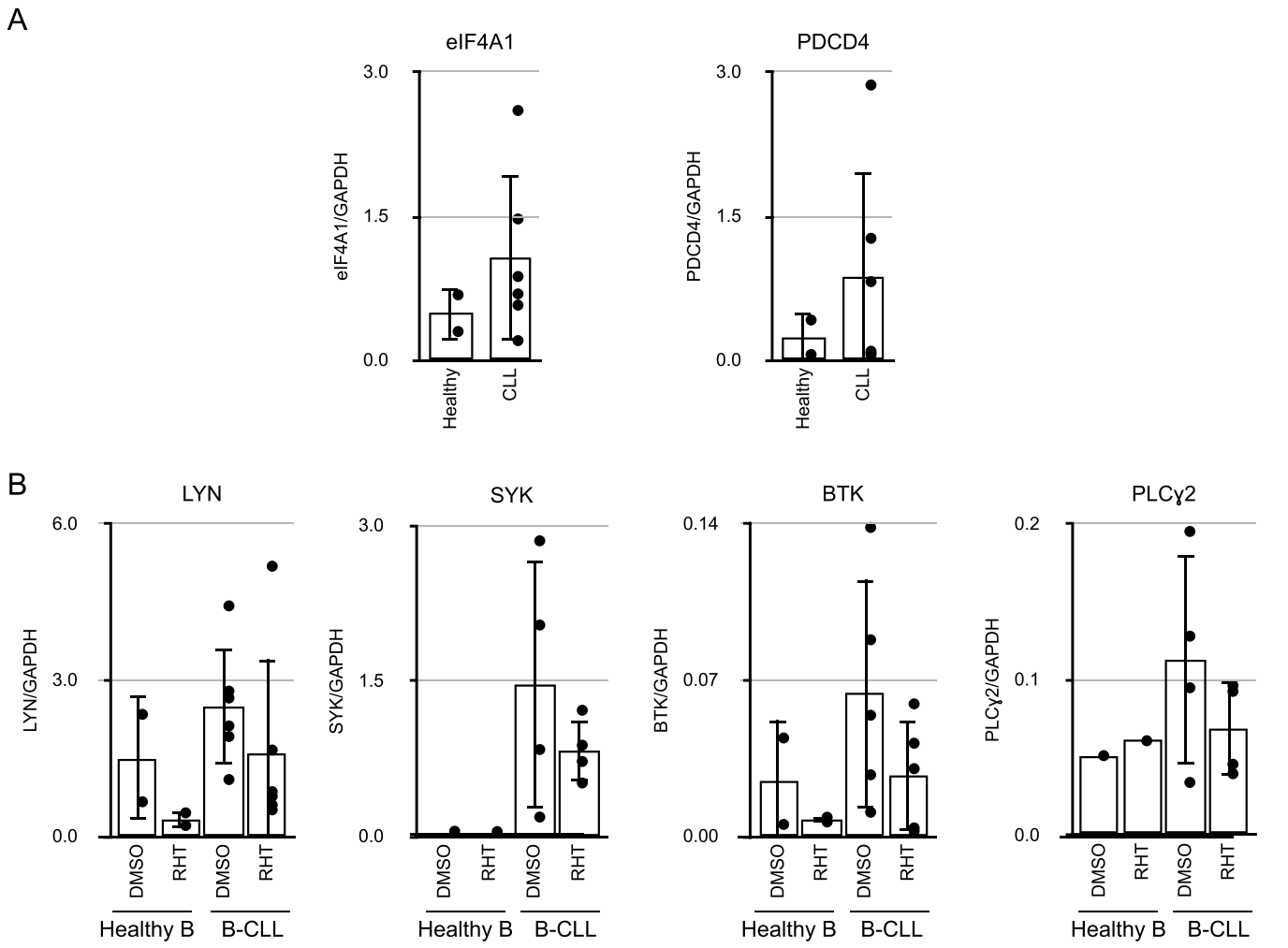


Fig. 3. B lymphocytes from CLL patients express higher eIF4A1 and PDCD4 protein levels. A. eIF4A1 and PDCD4 protein expression analysis in B lymphocytes extracts from healthy donors or CLL patients. Densitometric analyses of the bands relative to GAPDH expression and their standard deviations are depicted. Statistical significance was determined using Student's T-test ($p > 0.05$). B. The eIF4A1 inhibitor Rohinitib reduced the protein expression levels of BTK, LYN and PLCγ2 in B-CLL lymphocytes. B lymphocytes from CLL patients or healthy donors were treated ex vivo with 50 nM Rohinitib (RHT) for 16 h. Protein levels were determined by Western blot. A densitometric analysis of the immunoblots is represented. Statistical significance was determined using Student's T-test ($p > 0.05$).

3.4. Protein translation is a target of ubiquitin-like modifications in CLL

We also investigated whether NEDDylation had some regulatory influence on mRNA translation in CLL. In a di-glycine screening we found a vast representation of translation-related factors as over-modified in peripheral blood B-CLL cells, compared to B lymphocytes from healthy donors. Fig. 6A shows the variation in basal UBL modifications of translation initiation factors in B-CLL cells from two patients, relative to healthy B cells. Fig. 6B shows how MLN4924 alters such modifications. However, when we treated CLL peripheral mononuclear cells with MLN4924 ex vivo, no variation in the levels of eIF4A1 or PDCD4 proteins could be observed (Fig. 7A).

To test the effect that these UBL-PTM may have in the functionality of the translation machinery, we did an analysis of global translation by quantifying the incorporation of the puromycin analogue OPP. Basal translation in healthy CD19 + cells was lower than in CLL cells (Fig. 7B). As expected, Rohinitib significantly reduced the amount of nascent polypeptide chains, while MLN4924 had a limited effect, consistent with previous data [17]. However, when combined, MLN4924 potentiated Rohinitib action, further reducing general translation. When tested on CD19 + cells from healthy donors, this effect was not observed (Fig. 7B).

3.5. Transcriptome analysis

As an approach for the identification of the signaling pathways affected by the treatment with Rohinitib and MLN4924 we performed a whole transcriptome analysis in B-CLL cells from five CLL patients treated ex vivo with each of the compounds individually or in combination. A volcano plot of the variations in expression of apoptosis-related genes under the different treatments is shown in supplementary figure 5. A general overview of these plots allowed for the identification of two distinct groups of genes: One activated by the inhibition of NEDDylation (AKT1, AKT2, IRAK1) and another activated by the inhibition of eIF4A1 (NFKB1, PIK3R1, NFKBIA, MAP3K14, PRKAR2A, TNFRSF10B), neither of them much affected by the other treatment (supplementary figure 5, green and red, respectively). However, there were 9 genes which expression was cooperatively altered by the combination, that may explain the increase in cell death. Among them, the behavior of BCL2, TP53 or TNFRSF10B fitted well with the increase in apoptosis observed (Fig. 8). On the other hand, the expression of genes like ATM, APAF1, RELA or MAP3K14 seems contradictory to the induction of cell death. Supplementary Figure 6 displays additional cooperatively regulated genes categorized according to their involvement in two CLL phenotype-relevant pathways: NF-kB and WNT.

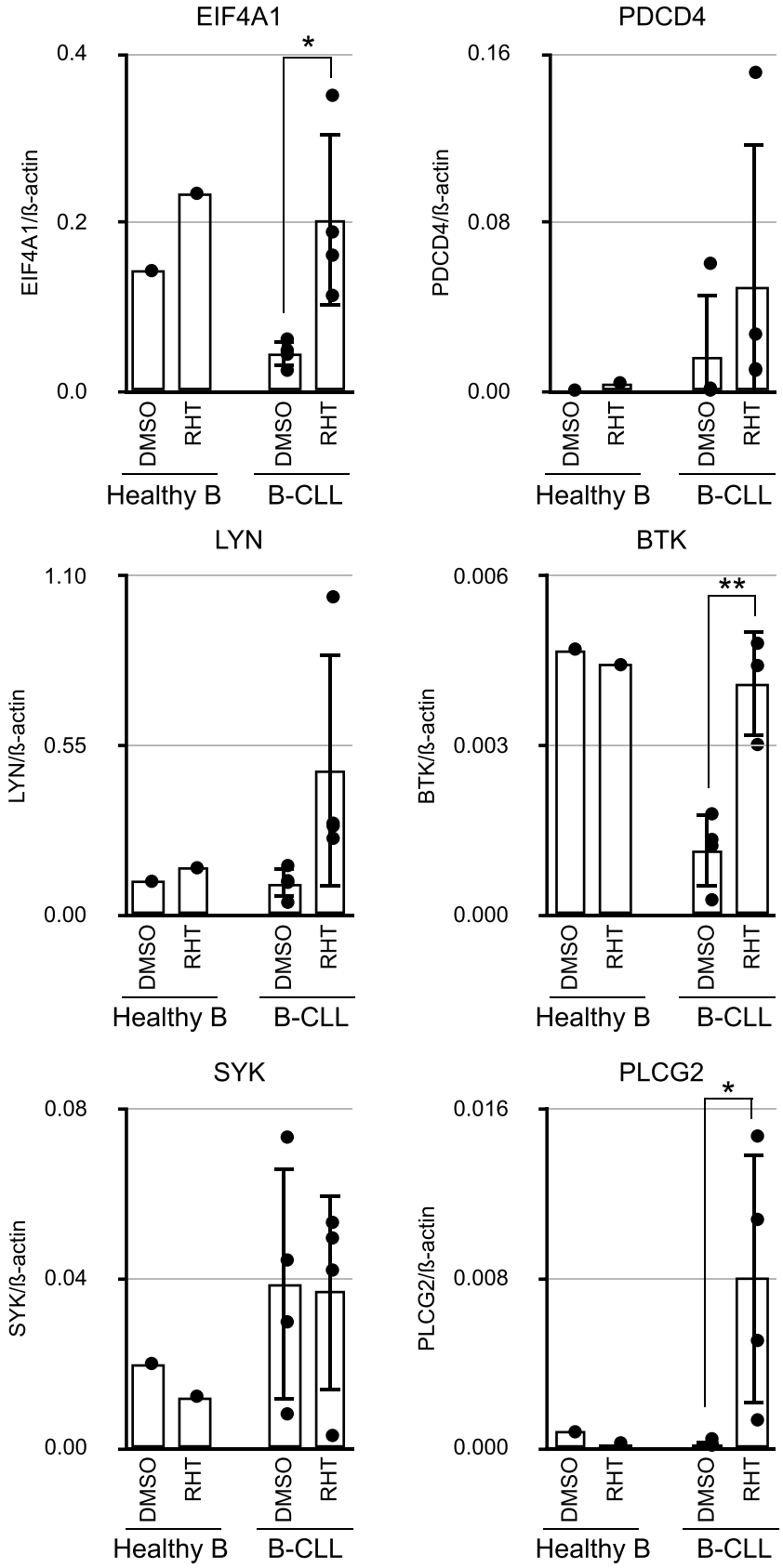


Fig. 4. Rohinitib stabilizes the mRNAs of several kinases involved in BCR signaling. Analysis of Rohinitib (RHT) action over the accumulation of the mRNAs of SYK, LYN, BTK and PLCG2 in healthy or CLL B cells by RT-qPCR. Expression levels relative to ß-actin mRNA and the standard deviations are depicted. Statistical significance was determined using Student́s T-test (* $p < 0.05$, ** $p < 0.01$).

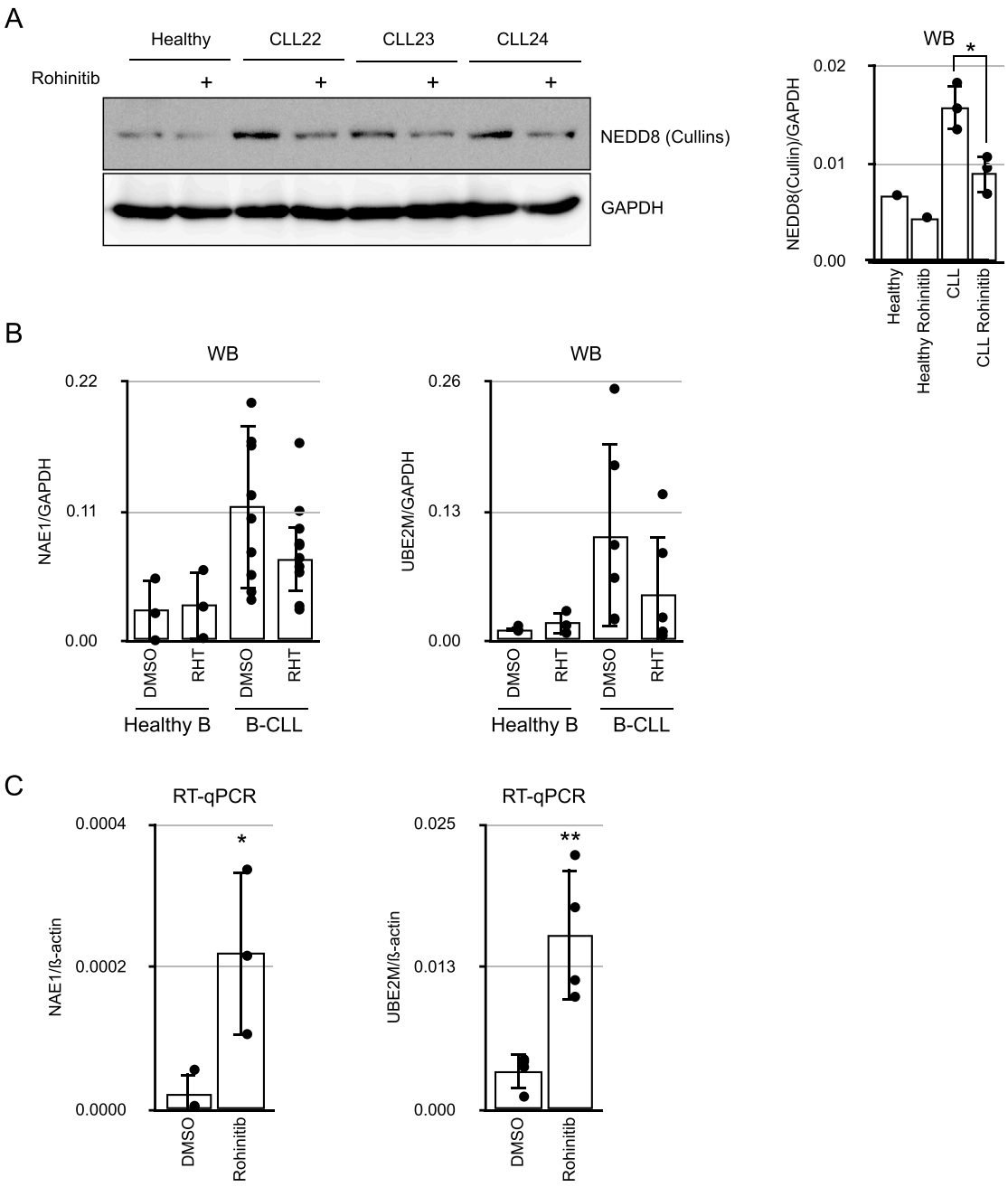


Fig. 5. Rohinitib reverts the elevated NEDDylation in CLL cells. A. Immunoblot analysis of the effect of 50 nM Rohinitib during 16 h over the elevated NEDDylation of Cullins in CLL cells. A densitometric analysis representing means and standard deviations is shown on the right. Statistical significance was determined using Student́s T-test (* $p = 0.019$). B. Analysis of Rohinitib action over the accumulation of the proteins NAE1 and UBE2M by immunoblot. Densistometric analysis of different samples relative to GAPDH protein expression and the standard deviation is represented. Ex vivo Rohinitib (RHT) treatment was as in A. Statistical significance was determined using Student́s T-test ($p = 0.087$ for NAE1, $p = 0.219$ for UBE2M, in CLL samples). C. RT-qPCR analysis of the mRNAs of B-CLL cells from different patients treated ex vivo with 50 nM Rohinitib for 16 h. The values of NAE1 or UBE2M mRNAs relative to ß-actin mRNA and the standard deviation are represented. Statistical significance was determined using Student́s T-test (* $p < 0.05$, ** $p < 0.01$).

4. Discussion

Chronic lymphocytic leukemia (CLL) is a multi-faceted malignancy, and several genomic and proteomic characteristics have been described since its inception. Some of these characteristics, such as constitutively active BCR signaling and the overexpression of BCL2, have demonstrated their value as therapeutic targets. However, the emergence of resistance and/or poor response necessitates the search for new therapeutic strategies. New therapies are now emerging to block these pathways in alternative ways. Some of them involve the targeted deletion of key proteins by promoting its ubiquitination and degradation at the proteasome [18]. Diverse BTK degraders are currently being tested for CLL treatment [19].

In their search for new therapeutic targets, several research groups have focussed their efforts in the altered proteostasis in CLL. In this context, it is noteworthy that CLL serves as a paradigmatic example of the dependency of certain tumors on the elevated expression of key proteins to maintain their transformation. Since these processes regulate the targets of many current CLL therapies, their inhibition may circumvent the emergence of mutations that often underlie therapy

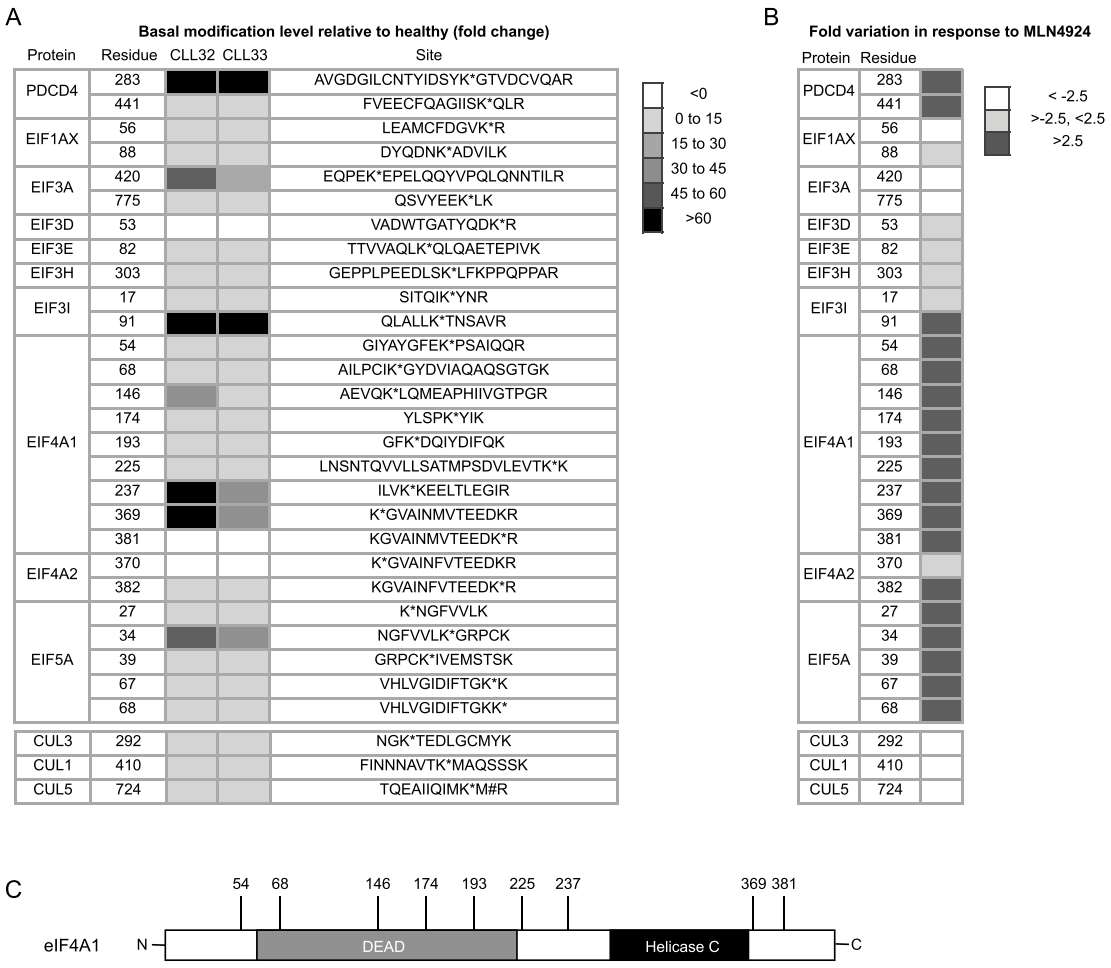


Fig. 6. Screening of the lysines aberrantly modified with UBL in CLL and their response to MLN4924. A. Heatmap of the UBL-PTMs of translational initiation factors in two CLL patients, relative to that of a pool of B lymphocytes from healthy donors (fold change). Data of characteristically NEDDylated lysines of Cullins are shown as reference. B. The right panel shows the mean variation (fold change) of the UBL-PTM after MLN4924 treatment. Data of characteristically NEDDylated lysines of Cullins are shown as reference. C. Schematic representation of the lysines of eIF4A1 with altered UBL modifications in CLL.

resistance [20]. RNA translation regulation is known to be altered in many tumors, and this is also applicable to CLL. Its potential therapeutic targeting is being explored [9].

On the other hand, our group recently described an elevation in ubiquitin like post-translational modifications in CLL [12,13]. NEDDylation has attracted special attention since the currently available inhibitor of NEDD8 activation, MLN4924/Pevonedistat, has demonstrated activity against B-CLL and other tumor cells [21]. As NEDDylation is a transversal cellular regulatory process, current experimental research and clinical trials are exploring its potential in combinational treatments [22].

In the present work we describe a synergic effect between MLN4924/Pevonedistat and the eIF4A1 translation factor inhibitor Rohinitib. The combination with MLN4924 virtually doubles the cytotoxic action of Rohinitib after 24-hour treatment, reaching a percentage of B-CLL cell death higher than 30 %. Cell death reaches levels close to 80 % after a 3-day treatment. Under the same conditions, the standard-of-care drug Ibrutinib induces around 7 % cell death after 24 h [11]. In our experiments, we kept MLN4924 concentration at 250 nM, in order to avoid collateral effects. In this respect, the combination of MLN4924 with Rohinitib exhibited a synergic effect, that may help in the elaboration of less toxic doses for its hypothetical application in humans. The effect seemed to be at least partially based on direct NEDDylations, as the ubiquitination inhibitor TAK243 did not show the same synergy with Rohinitib. Most importantly, this combinational treatment is not affecting healthy B cells, supporting a dysregulation of these translational and post-translational processes in CLL. We are preparing in vivo experiments to test this treatment in the Rosa26-RRAS2/mb1-Cre mouse model of CLL [23].

In order to shed some light on the molecular basis of such synergy, we explored the mutual interrelationships between these two processes altered in CLL. Despite the fact that many of the components of the RNA translation initiation, and in particular eIF4A1, exhibited an elevated level of UBL modifications in CLL, MLN4924 had no significant effect over the protein levels of the latter or its inhibitor, PDCD4. In accordance with previous results [17], MLN4924 on its own had no clear effect over the global RNA translation in B-CLL lymphocytes, although it slightly potentiated the inhibitory action of Rohinitib when added together to the cells. This effect was not observed in the global translation of B lymphocytes from healthy donors. A further study of the action of MLN4924 over other RNA translation initiation factors would be necessary to discriminate actions over specific mRNAs. It is noteworthy that NEDDylation promotes the stability of ribosomal proteins [24], so this might be another mechanism contributing to the discrepancies in the levels of certain RNAs and their encoded proteins in CLL cells.

We also explored the reciprocal regulation of NEDDylation by the control of the translation of its enzymes. eIF4A1 inhibition with Rohinitib induced a reduction in the NEDDylation of Cullins. Concurrently, Rohinitib reduced the protein levels of NAE1 and UBE2M while

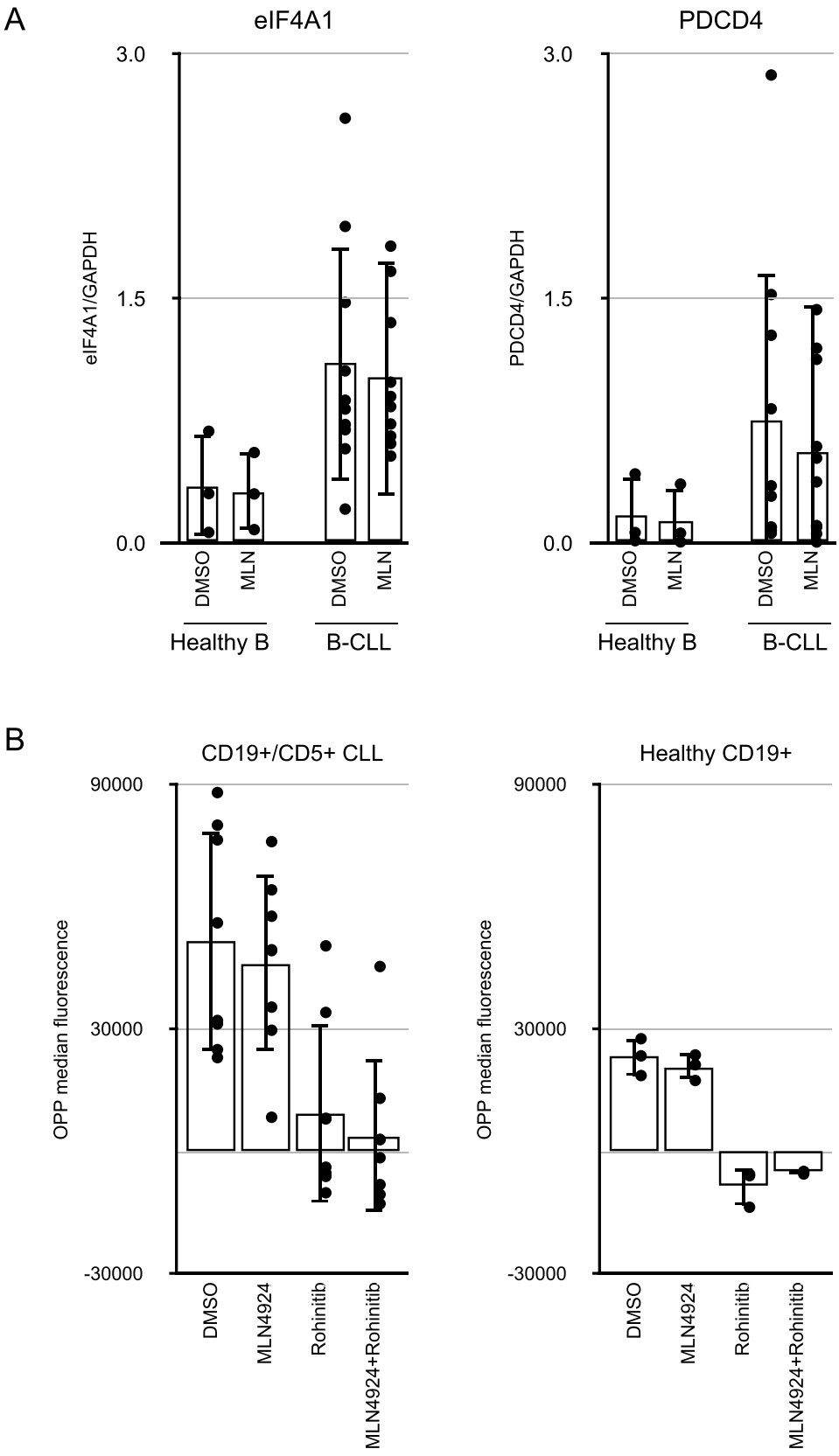


Fig. 7. Variation in global protein translation levels in response to Rohinitib and MLN4924. A. eIF4A1 and PDCD4 protein expression relative to GAPDH housekeeping in B lymphocytes from CLL patients or healthy donors treated with MLN4924 (MLN) or vehicle (DMSO). Densitometric analysis of 3 (healthy) or 10 (CLL) immunoblots are depicted. p-values were above 0.05 in a Student's T-test of the data. B. OPP incorporation assay to test the effect of 50 nM Rohinitib and/or 250 nM MLN4924 on global RNA translation. CLL pathologic (left, n = 8) and healthy (right, n = 3) B lymphocytes were tested ex vivo for 30 min. The OPP median fluorescence obtained after treatment with 30 μM cycloheximide for 30 min was subtracted from each value. Means and standard deviations are represented. p-values were above 0.05 in a Student's T-test of the Rohinitib + /- MLN4924 data.

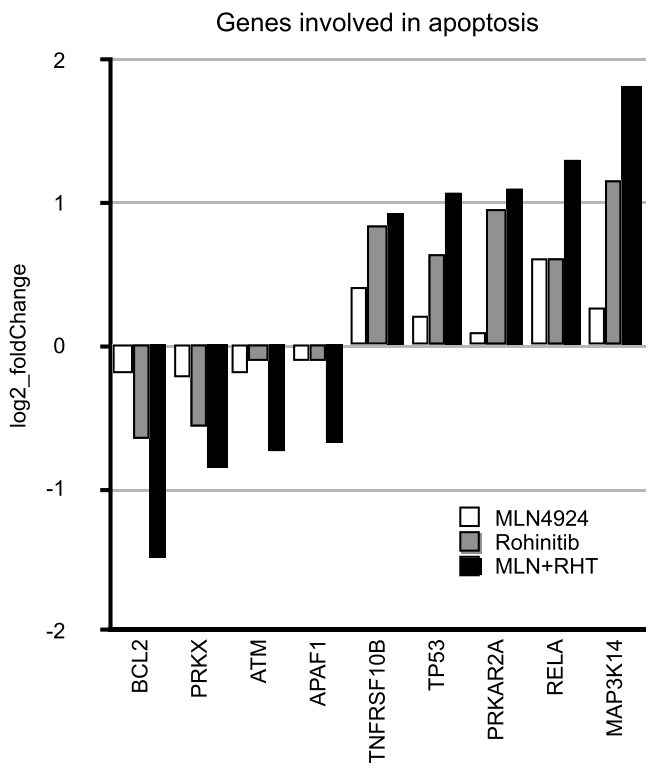


Fig. 8. Apoptosis-related genes with a cooperative expression variation in response to Rohinitib and MLN4924. PBMC from CLL patients (n = 5) were treated ex vivo for 16 h with 250 nM MLN4924, 50 nM Rohinitib or both (MLN+RHT) and their mRNA expression was analyzed by NGS.

protecting their mRNA from degradation, as has been shown to happen with other translation-regulated genes in CLL [7]. Moreover, Rohinitib also reverted the elevated protein expression of BCR signaling kinases, providing an explanation to their high accumulation in B-CLL lymphocytes but not that of their mRNAs. This may contribute to the constitutive activation of BCR signaling in B-CLL cells. These results situates the RNA translation alteration high in the hierarchy of events leading to CLL phenotype. Interestingly, a previous work reported the induction of eIF4A and eIF4GI, as well as the reduction of PDCD4, by anti-IgM stimulation of CLL cells [16].

To investigate the cellular processes affected by the combinational inhibition of NAE and eIF4A1, that may mediate their cooperative induction of apoptosis, we performed a transcriptome screening under each condition. The analysis detected genes regulated by the individual action of MLN4924 or Rohinitib. A group of genes were cooperatively regulated by the combined action of both drugs. Among them, the downregulation of BCL2 and the upregulation of TP53 stood out for their relevance in CLL [25,26]. BCL2 has been reported to be induced through NF-kB signaling. Indeed, NEDDylation has been reported to reduce IkB stability [27], and we have recently demonstrated the elevated translation of the NFKBIA mRNA in CLL cells [11], so the combined inhibition of both processes may explain BCL2 gene repression. On the other hand, p53 is known to repress BCL2 expression [28], and p53 NEDDylation inhibits its transcriptional activity [29]. Moreover, our previous data demonstrated how NEDDylation of p53 at lysine 120 competes with the acetylation of the latter, impairing p53 interaction with Bcl-2, as well as p53 transactivation activity [12]. Although all these data support our observations, it would be interesting to explore the exact mechanism by which these two drugs cooperatively induce TP53 mRNA accumulation and/or impairs BCL2 gene expression. In addition, further studies would be needed to delimitate the contribution of these genes to the induction of B-CLL lymphocyte death.

Ethics approval and consent to participate

Surpluses of peripheral blood samples from CLL patients were used after informed consent. Ethical approval was granted from the Ethics Committee of Cantabria (Resolution 2017.261).

Funding

V.A. and JL.C. are recipients of a contract from Instituto de Investigación Marqués de Valdecilla (IDIVAL).

CRediT authorship contribution statement

Víctor Arenas: Investigation. **Jose Luis Castaño:** Investigation. **Sara Fernández-Luis:** Methodology. **Juan José Domínguez:** Resources, Data curation. **Lucrecia Yáñez:** Supervision, Resources, Funding acquisition, Conceptualization. **Carlos Pipaón:** Writing – original draft, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank Amaya Llorente, Laura Álvarez and Patricia Maiso for the tests in multiple myeloma cell lines.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118398.

Data availability

Data will be made available on request.

References

[1] T.J. Kipps, F.K. Stevenson, C.J. Wu, et al., Chronic lymphocytic leukaemia, Published 2017 Jan 19, Nat. Rev. Dis. Prim. 3 (2017) 16096, https://doi.org/10.1038/nrdp.2016.96.
[2] X.S. Puente, S. Beà, R. Valdés-Mas, et al., Non-coding recurrent mutations in chronic lymphocytic leukaemia, Nature 526 (7574) (2015) 519–524, https://doi.org/10.1038/nature14666.
[3] A.M. Hortal, C.L. Oeste, C. Cifuentes, et al., Overexpression of wild type RRAS2, without oncogenic mutations, drives chronic lymphocytic leukemia, Published 2022 Feb 4, Mol. Cancer 21 (1) (2022) 35, https://doi.org/10.1186/s12943-022-01496-x.
[4] C. Wang, J. Leavenworth, C. Zhang, et al., Epigenetic regulation of EIF4A1 through DNA methylation and an oncogenic role of eIF4A1 through BRD2 signaling in prostate cancer, Oncogene 41 (19) (2022) 2778–2785, https://doi.org/10.1038/s41388-022-02272-3.
[5] A. Malina, J.R. Mills, J. Pelletier, Emerging therapeutics targeting mRNA translation, Published 2012 Apr 1, Cold Spring Harb. Perspect. Biol. 4 (4) (2012) a012377, https://doi.org/10.1101/cshperspect.a012377.
[6] Y. Nishida, R. Zhao, L.E. Heese, et al., Inhibition of translation initiation factor eIF4a inactivates heat shock factor 1 (HSF1) and exerts anti-leukemia activity in AML, Leukemia 35 (9) (2021) 2469–2481, https://doi.org/10.1038/s41375-021-01308-z.
[7] S. Wilmore, K.R. Rogers-Broadway, J. Taylor, et al., Targeted inhibition of eIF4A suppresses B-cell receptor-induced translation and expression of MYC and MCL1 in chronic lymphocytic leukemia cells, Cell Mol. Life Sci. 78 (17-18) (2021) 6337–6349, https://doi.org/10.1007/s00018-021-03910-x.
[8] A.L. Wolfe, K. Singh, Y. Zhong, et al., RNA G-quadruplexes cause eIF4A-dependent oncogene translation in cancer, Nature 513 (7516) (2014) 65–70, https://doi.org/10.1038/nature13485.
[9] V. Klapp, J. Paggetti, A. Largeot, E. Moussay, Targeting mRNA translation aberrations: a novel approach for therapy in chronic lymphocytic leukemia, Cancer Commun 43 (12) (2023) 1373–1376, https://doi.org/10.1002/cac2.12493.

[10] C. Paiva, J.C. Godbersen, T. Rowland, et al., Pevonedistat, a Nedd8-activating enzyme inhibitor, sensitizes neoplastic B-cells to death receptor-mediated apoptosis, Oncotarget 8 (13) (2017) 21128–21139, https://doi.org/10.18632/oncotarget.15050.
[11] V. Arenas, J.L. Castaño, J.J. Domínguez, L. Yáñez, C. Pipaón, Distinct NF-kB regulation favors a synergic action of pevonedistat and laduviglusib in B-chronic lymphocytic leukemia cells ex vivo, Published 2025 Feb 5, Cancers 17 (3) (2025) 533, https://doi.org/10.3390/cancers17030533.
[12] S. Bravo-Navas, L. Yáñez, Í. Romón, M. Briz, J.J. Domínguez-García, C. Pipaón, Map of ubiquitin-like post-translational modifications in chronic lymphocytic leukemia. Role of p53 lysine 120 NEDDylation, Leukemia 35 (12) (2021) 3568–3572, https://doi.org/10.1038/s41375-021-01184-7.
[13] V. Arenas, J.L. Castaño, J.J. Domínguez-García, L. Yáñez, C. Pipaón, A different view for an old disease: NEDDylation and other Ubiquitin-Like Post-Translational modifications in chronic lymphocytic leukemia, Published 2021 Sep 23, Front. Oncol. 11 (2021) 729550, https://doi.org/10.3389/fonc.2021.729550.
[14] H.M. Faessel, D.R. Mould, X. Zhou, D.V. Faller, F. Sedarati, K. Venkatakrishnan, Population pharmacokinetics of pevonedistat alone or in combination with standard of care in patients with solid tumours or haematological malignancies, Br. J. Clin. Pharm. 85 (11) (2019) 2568–2579, https://doi.org/10.1111/bcp.14078.
[15] A. Ianevski, A.K. Giri, T. Aittokallio, SynergyFinder 3.0: an interactive analysis and consensus interpretation of multi-drug synergies across multiple samples, Nucleic Acids Res. 50 (W1) (2022) W739–W743, https://doi.org/10.1093/nar/gkac382.
[16] A. Yeomans, S.M. Thirdborough, B. Valle-Argos, et al., Engagement of the B-cell receptor of chronic lymphocytic leukemia cells drives global and MYC-specific mRNA translation, Blood 127 (4) (2016) 449–457, https://doi.org/10.1182/blood-2015-07-660969.
[17] T.A. Soucy, P.G. Smith, M.A. Milhollen, et al., An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer, Nature 458 (7239) (2009) 732–736, https://doi.org/10.1038/nature07884.
[18] Z. Liu, M. Hu, Y. Yang, et al., An overview of PROTACs: a promising drug discovery paradigm, Published 2022 Dec 20, Mol. Biomed. 3 (1) (2022) 46, https://doi.org/10.1186/s43556-022-00112-0.
[19] R.T. Salvaris, J. Brennan, K.L. Lewis, BTK is the target that keeps on giving: a review of BTK-Degrader drug development, clinical data, and future directions in CLL, Published 2025 Feb 6, Cancers 17 (3) (2025) 557, https://doi.org/10.3390/cancers17030557.
[20] A. Wiegering, F.W. Uthe, T. Jamieson, et al., Targeting translation initiation bypasses signaling crosstalk mechanisms that maintain high MYC levels in colorectal cancer, Cancer Discov. 5 (7) (2015) 768–781, https://doi.org/10.1158/2159-8290.CD-14-1040.
[21] D.J. Fu, T. Wang, Targeting NEDD8-activating enzyme for cancer therapy: developments, clinical trials, challenges and future research directions, Published 2023 Jul 31, J. Hematol. Oncol. 16 (1) (2023) 87, https://doi.org/10.1186/s13045-023-01485-7.
[22] P. Torka, S.K. Thiruvengadam, L. Chen, et al., Pevonedistat, a Nedd8-activating enzyme inhibitor, in combination with ibrutinib in patients with relapsed/refractory B-cell non-Hodgkin lymphoma, Published 2023 Jan 11, Blood Cancer J. 13 (1) (2023) 9, https://doi.org/10.1038/s41408-022-00763-w.
[23] A.M. Hortal, A. Villanueva, I. Arellano, et al., Mice overexpressing Wild-Type RRAS2 are a novel model for preclinical testing of anti-chronic lymphocytic leukemia therapies, Published 2023 Dec 12, Cancers 15 (24) (2023) 5817, https://doi.org/10.3390/cancers15245817.
[24] D.P. Xirodimas, A. Sundqvist, A. Nakamura, L. Shen, C. Botting, R.T. Hay, Ribosomal proteins are targets for the NEDD8 pathway, EMBO Rep. 9 (3) (2008) 280–286, https://doi.org/10.1038/embor.2008.10.
[25] M. Hanada, D. Delia, A. Aiello, E. Stadtmauer, J.C. Reed, bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia, Blood 82 (6) (1993) 1820–1828.
[26] I. Cordone, S. Masi, F.R. Mauro, et al., p53 expression in B-cell chronic lymphocytic leukemia: a marker of disease progression and poor prognosis, Blood 91 (11) (1998) 4342–4349.
[27] J.C. Godbersen, C. Paiva, O.V. Danilova, A. Berger, J.R. Brown, A.V. Danilov, Targeting neddylation effectively antagonizes nuclear factor-κB in chronic lymphocytic leukemia B-cells, Leuk. Lymphoma 56 (5) (2015) 1566–1569, https://doi.org/10.3109/10428194.2014.990901.
[28] Y. Wu, J.W. Mehew, C.A. Heckman, M. Arcinas, L.M. Boxer, Negative regulation of bcl-2 expression by p53 in hematopoietic cells, Oncogene 20 (2) (2001) 240–251, https://doi.org/10.1038/sj.onc.1204067.
[29] D.P. Xirodimas, M.K. Saville, J.C. Bourdon, R.T. Hay, D.P. Lane, Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity, Cell 118 (1) (2004) 83–97, https://doi.org/10.1016/j.cell.2004.06.016.