Electron transport chain and mTOR inhibition synergistically decrease CD40 signaling and counteract venetoclax resistance in chronic lymphocytic leukemia

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Abstract

CD40 signaling upregulates BCL-XL and MCL-1 expression in the chronic lymphocytic leukemia (CLL) lymph node microenvironment, affording resistance to the BCL-2 inhibitor, venetoclax. Venetoclax resistance in the therapeutic setting and after long-term laboratory selection has been linked to metabolic alterations, but the underlying mechanism(s) are unknown. We aimed here to discover how CD40 stimulation as a model for tumor microenvironment-mediated metabolic changes, affects venetoclax sensitivity/resistance. CD40 stimulation increased oxidative phosphorylation and glycolysis, but only inhibition of oxidative phosphorylation countered venetoclax resistance. Furthermore, blocking mitochondrial import of pyruvate, glutamine or fatty acids affected CLL metabolism, but did not prevent CD40-mediated resistance to venetoclax. In contrast, inhibition of the electron transport chain (ETC) at complex I, III or V attenuated CLL activation and ATP production, and downregulated MCL-1 and BCL-XL, correlating with reduced CD40 surface expression. Moreover, ETC inhibition equaled mTOR1/2 but not mTOR1 inhibition alone for venetoclax resistance, and all three pathways were linked to control of general protein translation. In line with this, ETC plus mTOR inhibition synergistically counteracted venetoclax resistance. These findings link oxidative CLL metabolism to CD40 expression and cellular signaling, and may hold clinical potential.

Introduction

Chronic lymphocytic leukemia (CLL) has become a paradigm for cancer in which the microenvironment plays a key role in the physiopathology of the disease.[1-3] CLL cells circulate between lymph node (LN) niches and peripheral blood, which confer proliferating and quiescent states, respectively. CLL cells receive various signals in the LN microenvironment, which contains macrophages, stromal cells, monocyte-derived nurse-like cells and T cells. These signals promote cell survival, growth, proliferation and trafficking of cells between the peripheral blood and LN. The microenvironment in the LN activates and protects CLL cells through several mechanisms, such as chemokines, CLL surface molecules, adhesion molecules and tumor necrosis factor receptor members, which protect CLL cells from apoptosis.[4-6] Among these mechanisms, the interactions between CLL and $CD4^+$ T helper cells via CD40-CD40L play a significant role in contributing to resistance to apoptosis.[7-11] The expression of anti-apoptotic proteins BCL-XL and MCL-1 in CLL cells from LN samples is higher than that in CLL cells from peripheral blood.[12] We and others also found that *in vitro* CD40 stimulation increases the expression of BCL-XL, MCL-1 and Bfl-1, thereby mimicking LN signaling,[13,14] which is important for the resistance of CLL to the widely used BCL-2 inhibitor venetoclax.[11,13] In line with this, recent clinical trials show that in contrast to responses in the blood, LN responses are less complete.[15] Hence, remaining LN sites are a likely source of emerging resistance, which is a growing clinical problem.[16,17] Importantly, changes in cellular energy metabolism and mitochondrial reprogramming have also been linked to resistance to venetoclax,[18-20] but the underlying mechanism(s) are still unknown.

The well-known Warburg effect holds that aerobic glycolysis, i.e., limiting energy metabolism largely to glycoly-

sis even in the presence of oxygen, is a hallmark of cancer.[21,22] However, CLL does not behave metabolically like other "Warburg malignancies", since enhanced mitochondrial oxidative phosphorylation (OXPHOS) but not glycolysis was seen in circulating CLL cells as compared to healthy B cells.[23] In addition, we recently reported that CLL cells in LN have higher mitochondrial mass and glucose uptake than CLL cells in the blood, and that *in vitro* B-cell receptor and CD40 stimulation recapitulates various metabolic alterations observed in LN cells.[24] The enhanced glycolysis and OXPHOS pathways were confirmed on gene expression and cellular metabolic levels. An important finding was that amino acids, in particular glutamine, fuel the mitochondrial tricarboxylic acid (TCA) cycle and thereby drive OXPHOS, while the contribution of glucose to OXPHOS is much lower.[24] Even in cancers driven by the Warburg effect, it has been demonstrated that mitochondrial OXPHOS is crucial for survival.[25-27] OXPHOS promotes the generation of metabolites for nucleotide, lipid and amino acid synthesis, which are essential for cell proliferation, yet it also generates onco-metabolites that contribute to tumorigenesis.[28,29]

As mentioned above, induced venetoclax resistance in cell lines has been associated with increased OXPHOS, and metabolic modulators can cooperate with venetoclax to overcome resistance.[18] However, the link between OXPHOS and sensitivity to venetoclax in relationship to primary CLL in the microenvironment remains unclear. Therefore, using CD40 signaling as a common denominator, we investigated if and how energy metabolism is associated with venetoclax resistance in primary human CLL cells.

Methods

Patients' material and reagents

Material was obtained from CLL patients, after having obtained their written informed consent, during routine follow-up or diagnostic procedures at the Academic Medical Center, Amsterdam, the Netherlands. The studies were approved by our Ethical Review Board and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral blood mononuclear cells from patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands) were cryopreserved and stored in liquid nitrogen. All samples contained more than 85% $CD5^+CD19^+$ cells, as assessed by flow cytometry. More details on the patients are provided in *Online Supplementary Table S1*. All reagents and products used are listed in *Online Supplementary Table S2*.

Cell culture, flow cytometry and the venetoclax sensitivity assay, microarrays, microarray data normalization and differential expression, metabolic assays, western blot analysis, real-time polymerase chain reaction, and the protein translation assay are described in detail in the *Online Supplementary Data*.

Statistics

The Student *t* test was used to analyze paired observations. One-way analysis of variance with multiple testing corrections was used to analyze differences between groups. The specific statistic test applied is indicated in the figure legends. For the figures to display relative values normalized to the condition of growth on 3T3 fibroblasts, statistical analysis was performed on data that were normalized. Statistically significant data are indicated as $*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$.

Results

Oxidative phosphorylation contributes to the resistance of primary chronic lymphocytic leukemia cells to venetoclax

We previously showed that CD40 activation mimics the metabolic conditioning of CLL in the tumor microenvironment (TME).[24] We corroborated this further by analyzing the oxygen consumption rate and extracellular acidification rate, as indicators of OXPHOS and glycolytic activity, respectively, using a Seahorse flux analyzer. After 48 h of CD40 stimulation *in vitro*, CLL cells showed higher oxygen consumption rate at both the basal and maximal levels (*Online Supplementary Figure S1A, B*), indicating that OXPHOS was increased. The same occurred with glycolysis, as CD40 activation increased extracellular acidification rate (*Online Supplementary Figure S1C*). Secondly, increased OXPHOS and glycolysis were further validated by analyzing our previously published microarray dataset.[30] Accordingly, the majority of genes involved in OXPHOS and glycolysis were upregulated by CD40 stimulation (Figure 1A, B). In addition, pathway analysis confirmed that both OXPHOS and glycolysis were significantly upregulated by CD40 signaling, with both of them ranking among the top eight pathways (Figure 1C).

To investigate whether these major changes in metabolic pathways are linked to venetoclax resistance, CLL cells were treated with OXPHOS or glycolysis inhibitors during CD40 stimulation and subsequently exposed to venetoclax. OXPHOS inhibition by oligomycin substantially counteracted venetoclax resistance (1,000-fold shift in IC_{50}), while glycolysis inhibition by 2-deoxy-D-glucose had a very modest, non-significant effect (Figure 1D). The addition of venetoclax did not have a direct effect on oxygen consumption rate (*Online Supplementary Figure S1D*). Of note, neither inhibition of OXPHOS nor of glycolysis directly induced apoptosis of CLL cells, although 2-deoxy-D-glucose reduced the pro-survival effect of CD40

stimulation back to the unstimulated level (Figure 1E). In conclusion, inhibition of OXPHOS/ATPase by oligomycin during CD40 stimulation did not affect CLL viability, but rapidly and strongly attenuated induction of venetoclax resistance.

Inhibition of glutaminolysis impairs chronic lymphocytic leukemia cell metabolism but not sensitivity to venetoclax

ATP is produced by OXPHOS via the electron transport chain (ETC). The TCA cycle supplies FADH2 and NADH, which subsequently transfer their electrons to the ETC.[31] To further investigate the cross-talk between CD40 signaling, resistance to venetoclax and mitochondrial metabolism, we studied the role of the TCA cycle. To do this, we inhibited import of the three main fuels into the mitochondria and TCA cycle was inhibited alone or in combination during CD40 stimulation: pyruvate was blocked by UK5099, glutamine by 6-diazo-5-oxo-L-norleucine (DON), and fatty acids by etomoxir. The increase in mitochondrial mass observed upon CD40 stimulation was strongly reduced by oligomycin and at best marginally affected (e.g., DON) by these three fuel inhibitors (Figure 2A). Increased glucose uptake follow-

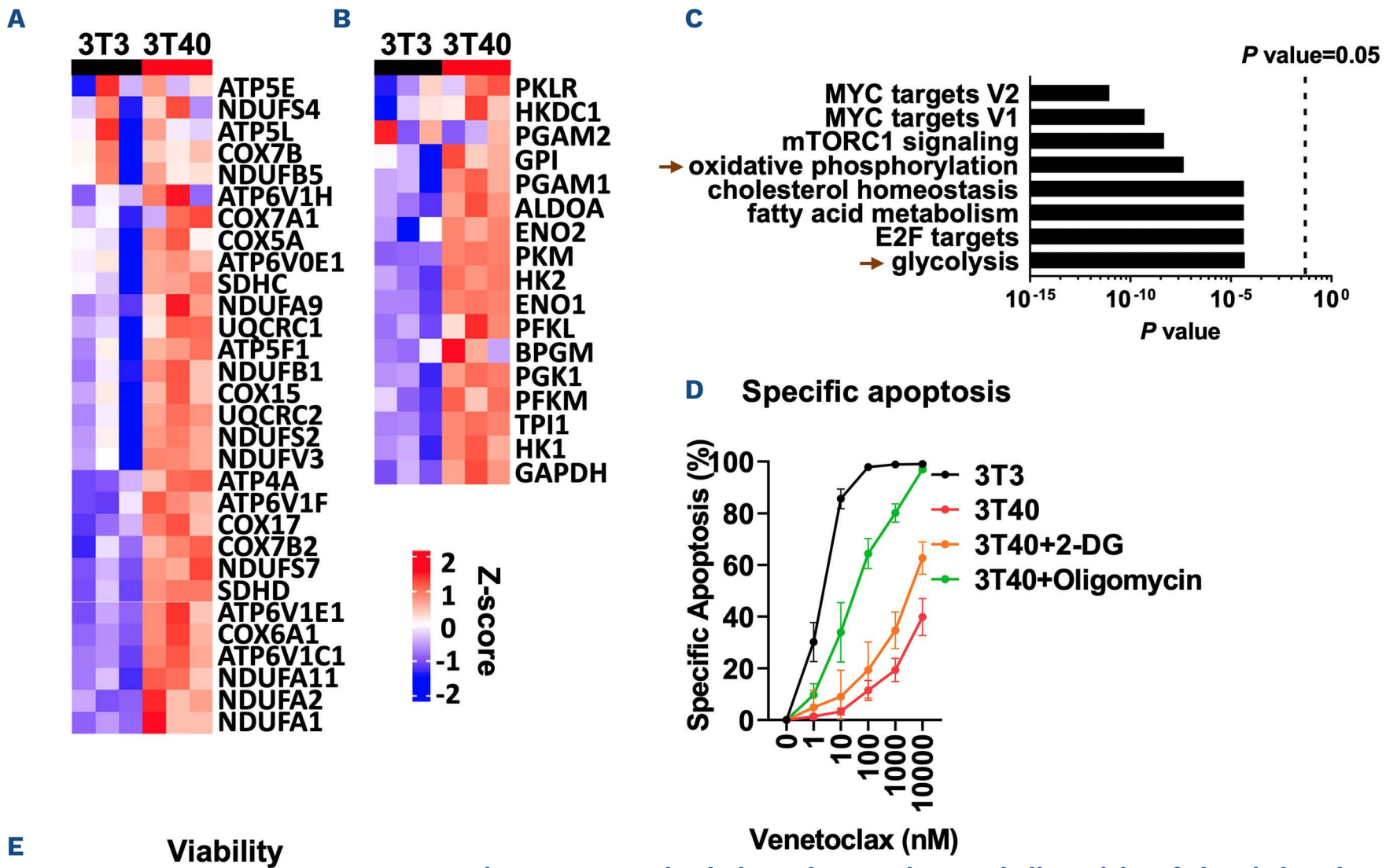

Figure 1. CD40 stimulation enhances the metabolic activity of chronic lymphocytic leukemia cells, and blocking oxidative phosphorylation but not glycolysis attenuates resistance to venetoclax. Gene expression data were retrieved from a previously published microarray dataset of chronic lymphocytic leukemia (CLL) cells cultured on 3T3 or 3T40 fibroblasts for 16 h.[30] (A) Heatmap of the expression of the top 30 significantly changed oxidative phosphorylation-related genes by 3T40 (N=3). (B) Heatmap of the expression of glycolysis-related genes by 3T40 (N=3). (C) Top eight pathways upregulated by 3T40. Pathway analysis was performed using CAMERA.[60] Gene sets were retrieved from MSigDB v5.1 (hallmark gene sets, Entrez Gene ID): *http://www.broadinstitute.org/gsea/msigdb/index.jsp*. (D) CLL cells (N=6) were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with 10 nM oligomycin or 4 μM 2-deoxy-D-glucose for 24 h. Subsequently, CLL cells were harvested and incubated with a titration of 1-10,000 nM venetoclax for another 24 h after which viability was measured using DiOC6 and TO-PRO-3. Specific apoptosis was calculated. (E) Viability of CLL cells was also measured by flow cytometry directly after harvesting from fibroblasts. Data are shown as mean ± standard error of mean. One-way analysis of variance was performed to compare culture on 3T3 fibroblasts to the other conditions. Each patient is labeled with a distinct symbol across all the conditions. 2-DG: 2-deoxy-D-glucose.

ing CD40 stimulation was attenuated by oligomycin and DON, but not by either UK-5099 or etomoxir (Figure 2A). In addition, DON clearly suppressed both basal oxygen consumption rate and extracellular acidification rate, whereas the other two fuel inhibitors, UK5099 and etomoxir, had much weaker effects (Figure 2B). The combination of UK5099 and etomoxir did not have a significant impact on metabolic parameters (*Online Supplementary Figure S2A, B*), and only the combinations including DON resulted in significant decreases of glycolytic and mitochondrial parameters (*Online Supplementary Figure S2A, B*). Importantly, none of the combinations decreased CLL cell viability (*Online Supplementary Figure S2C*). These results indicate that inhibition of glutamine conversion to glutamate by DON is key for the maintenance of CLL metabolism, rather than pyruvate or long-chain fatty acids. This is in accordance with our previous findings that CLL cells predominantly use glutamine/glutamate to fuel the TCA cycle.[24] Although the fuel inhibitors had an impact on CLL metabolic activity, none of them affected CD40 stimulation itself, represented by CD95 induction. The induction of CD95 remained stable after single or combinations of inhibition of the three main fuels of the TCA cycle, while it was significantly decreased by the OXPHOS inhibitor oligomycin (Figure 2C, *Online Supplementary Figure S2D*). Most importantly, venetoclax resistance was strongly affected by oligomycin as before (Figure 1D), yet hardly affected, not even by DON, by any of the fuel inhibitors (Figure 2D, *Online Supplementary Figure S2E*). As previously indicated, CLL cells showed high metabolic flexibility when OXPHOS was inhibited; after CLL cells were treated with oligomycin, oxygen consumption rate was largely decreased while extracellular acidification rate was elevated (Figure 2B). This suggested that CLL cells could easily switch to glycolysis as their main energy path-

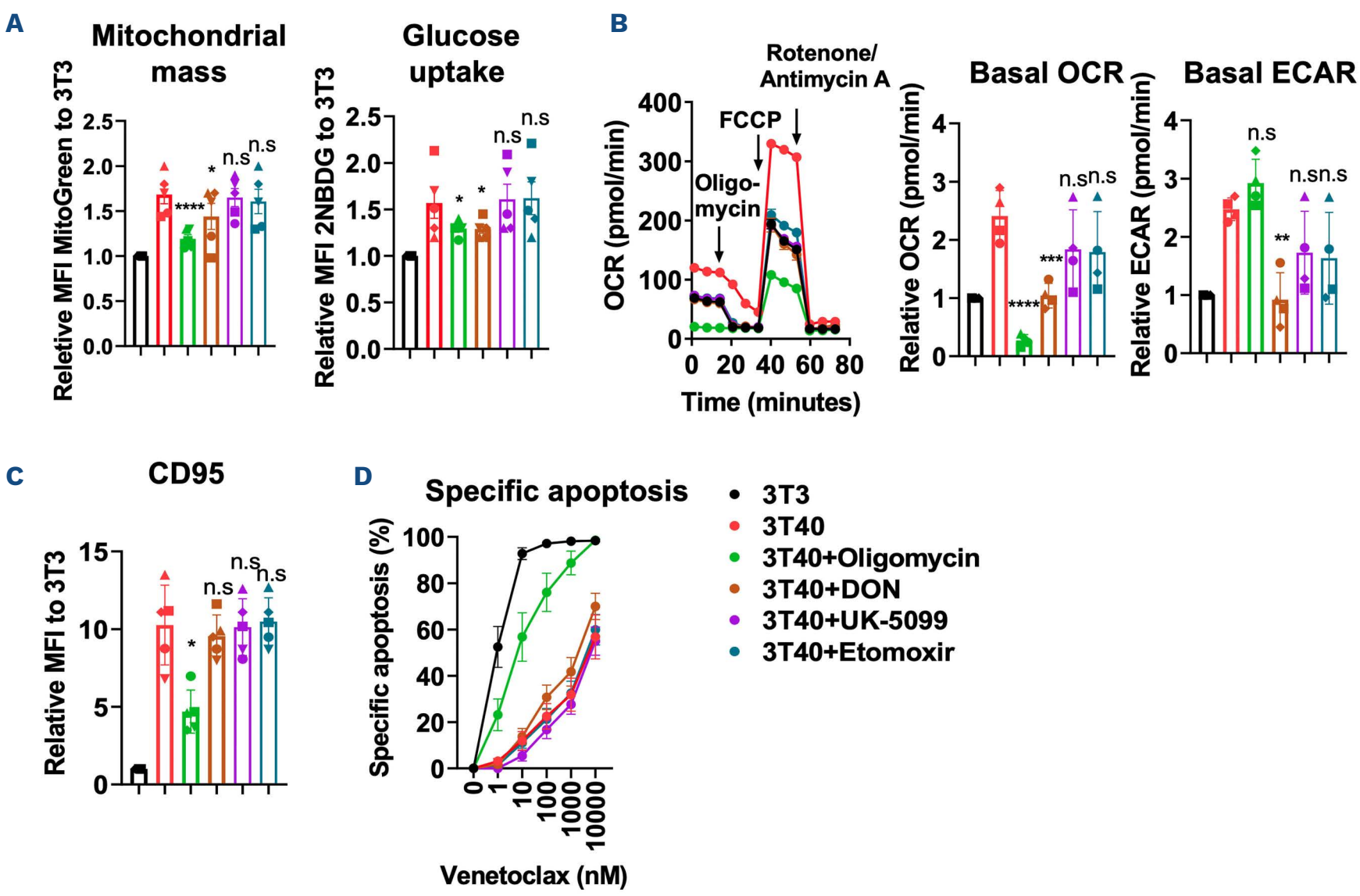

Figure 2. Inhibition of glutaminolysis impairs chronic lymphocytic leukemia cell metabolism but not sensitivity to venetoclax. Chronic lymphocytic leukemia (CLL) cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with 10 nM oligomycin, 20 μM 6-diazo-5-oxo-L-norleucine, 2 μM UK-5099 or 4 μM etomoxir for 24 h. After resuspension, (A) mitochondrial mass and glucose uptake were measured by flow cytometry (N=5). (B) A Seahorse MitoStress test was performed (N=4), basal oxygen consumption rate and basal extracellular acidification rate were measured on a Seahorse XF96 analyzer. (C) CD95 expression was measured by flow cytometry (N=5). (D) Cells were subsequently incubated with a titration of 1-10,000 nM venetoclax for 24 h after which viability was measured using DiOC6 and TO-PRO-3 staining. Then specific apoptosis was calculated (N=5). Data are shown as mean ± standard error of mean. One-way analysis of variance was performed to compare culture on 3T40 fibroblasts to the other conditions. Each patient is labeled with a distinct symbol across all the conditions. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n.s.: not significant. MFI: mean fluorescence intensity; OCR: oxygen consumption rate; FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; ECAR: extracellular acidification rate; DON: 6-diazo-5-oxo-L-norleucine.

way when OXPHOS was inhibited. In contrast, DON decreased OXPHOS of CLL cells, but to a lesser extent than oligomycin, and apparently did not enforce CLL cells to enhance lactate production (Figure 2B). Thus, inhibition of import of these three fuels was not capable of affecting venetoclax sensitivity, despite attenuation of OXPHOS. These data suggested that either other (not tested) substrates also contribute to fueling the TCA cycle in CD40-stimulated CLL cells or that oligomycin has additional effects besides ETC inhibition. We continued our search by investigating the latter option.

Electron transport chain inhibitors interfere with CD40 activation and anti-apoptotic protein expression

As oligomycin inhibits the mitochondrial complex V, the last stage of the ETC, we studied whether resistance to venetoclax could also be overcome by inhibiting the activity of the other ETC complexes, using inhibitors of complex I (rotenone), II (dimethyl malonate) and III (antimycin A) of OXPHOS. These mitochondrial complexes were blocked by inhibitors during CD40 activation, which had no direct effect on CLL viability (*Online Supplementary Figure S3A*). Complex I and III inhibitors (rotenone, antimycin A) also increased sensitivity to venetoclax, but to a lesser extent than oligomycin (Figure 3A). ATP levels were decreased upon inhibition of complexes I, III and V (*Online Supplementary Figure S3B*). In contrast, inhibiting complex II of the ETC with dimethyl malonate had no effect on venetoclax sensitivity and also did not decrease ATP levels (*Online Supplementary Figure S3B*). These data indicate that the specific inhibition of complexes involved in proton pumping and ATP production leads to venetoclax sensitivity. Except for the complex II inhibitor, all other ETC inhibitors decreased CD95 expression to similar levels (Figure 3B).

Venetoclax resistance is directly correlated with the expression levels of anti-apoptotic proteins MCL-1 and BCL-XL.[13,14] Indeed, the CD40-induced proteins BCL-XL and MCL-1 were strongly downregulated by all ETC inhibitors except for dimethyl malonate (Figure 3C). These analyses were done by flow cytometry, as described before;[14,32] details are provided in the *Online Supplementary Methods* and *Online Supplementary Figure S3C*. BCL-2 expression was altered in a opposite way (Figure 3C), indicating that not all pro-survival proteins were similarly affected. OXPHOS inhibition can lead to increased production of reactive oxygen species, which can affect cell survival pathways,[33,34] but levels of total or mitochondrial reactive oxygen species levels upon ETC inhibition were not affected by oligomycin (*Online Supplementary Figure S3D*). Additionally, N-acetyl-L-cysteine, a reactive oxygen species scavenger, was utilized in conjunction with rotenone, antimycin A and oligomycin. The results demonstrated that the decrease in CD95 expression induced by these ETC inhibitors was not recovered but even further reduced by N-acetyl-L-cysteine, while viability was preserved (Figure 3D). Since CD40 signaling was affected by ETC inhibition, we investigated overall expression of CD40 protein and RNA expression (Figure 3E, F). Interestingly, CD40 stimulation had a huge impact on CD40 expression itself: RNA expression increased more than 300-fold (Figure 3F). Protein expression also increased, but not as dramatically as that of the RNA. Importantly, these changes were blocked by ETC inhibition, except for inhibition of complex II (Figure 3E). These findings indicate that ETC inhibition at complex I, III or V reduces CD40 signaling and venetoclax resistance in CLL, while oxidation of succinate to fumarate by complex II is not involved in this process. Furthermore, these effects are not due to overproduction of reactive oxygen species.

mTOR connects oxidative phosphorylation with CD40 signaling and venetoclax resistance

We next investigated how CD40 downstream kinases link CD40 signaling, metabolism and venetoclax resistance. We therefore inhibited phosphatidyl inositol 3-kinase (PI3K) by idelalisib, Bruton tyrosine kinase (BTK) by ibrutinib, mTOR1 by rapamycin, mTOR1/2 by AZD8055 and protein kinase B (AKT) by MK2206 (*Online Supplementary Figure S4A*). The results showed that besides oligomycin, only AZD8055 decreased mitochondrial mass (Figure 4A, *Online Supplementary Figure S4B*), and both rapamycin and AZD8055, but not the others, modestly suppressed glucose uptake (Figure 4A, *Online Supplementary Figure S4B*). mTOR inhibition, especially by AZD8055, decreased basal oxygen consumption rate, extracellular acidification rate and spare respiration capacity, while inhibition of BTK or PI3K did not (Figure 4B). Importantly, AZD8055, but not rapamycin or other kinase inhibitors, suppressed the overexpression of the anti-apoptotic proteins BCL-XL and MCL-1 by CD40 signaling (Figure 4C, *Online Supplementary Figure S4C*), which consequently strongly alleviated resistance to venetoclax (Figure 5A, *Online Supplementary Figure S4D*). These results suggested that the effects of ETC inhibition on CD40 signaling might be analogous to those of inhibition of mTOR and, more prominently, both mTOR1 and mTOR2 by AZD8055.

Since AZD8055 showed similar effects as ETC inhibitors, we checked CD40 expression and activation upon mTOR inhibition. Figure 5B shows that only AZD8055, like oligomycin, clearly suppressed the expression of CD40 itself and the activation marker CD95 (Figure 5C). In addition, the quantification data indicated that the effects of rapamycin on CD40 expression were quite variable across patients. Importantly, rapamycin as a single agent did not affect CD40-induced resistance to venetoclax (Figure 5A). To further probe the relationship between mTOR and OXPHOS, several downstream targets of mTOR were evaluated in the presence of oligomycin in comparison with AZD8055 or rapamycin. CD40 activation induced p-AKT^{S473}, p-GSK3β,^{S9} p-eIF2α^{S51} and p-AKT^{T450}, while oligomycin,

AZD805 and rapamycin suppressed these important downstream effectors (*Online Supplementary Figure S5*). There were differences in terms of quantification of these phosphorylation events, which can be expected when analyzing primary patients' samples, but the overall pattern was consistent. Of note, ibrutinib and idelalisib as upstream inhibitors of BTK and PI3K did not have effects (*Online Supplementary Figure S5*). In conclusion, the compiled data indicate that inhibition of mTOR1/2 had equivalent effects on metabolism and venetoclax sensitivity as ETC inhibition by oligomycin.

Electron transport chain and mTOR inhibition suppress general protein translation and are synergistic in reversing resistance to venetoclax

AKT and mTOR1/signaling have an important effect on translation, including the regulation of expression of specific pro-survival proteins in the context of CLL.[30,35] There-

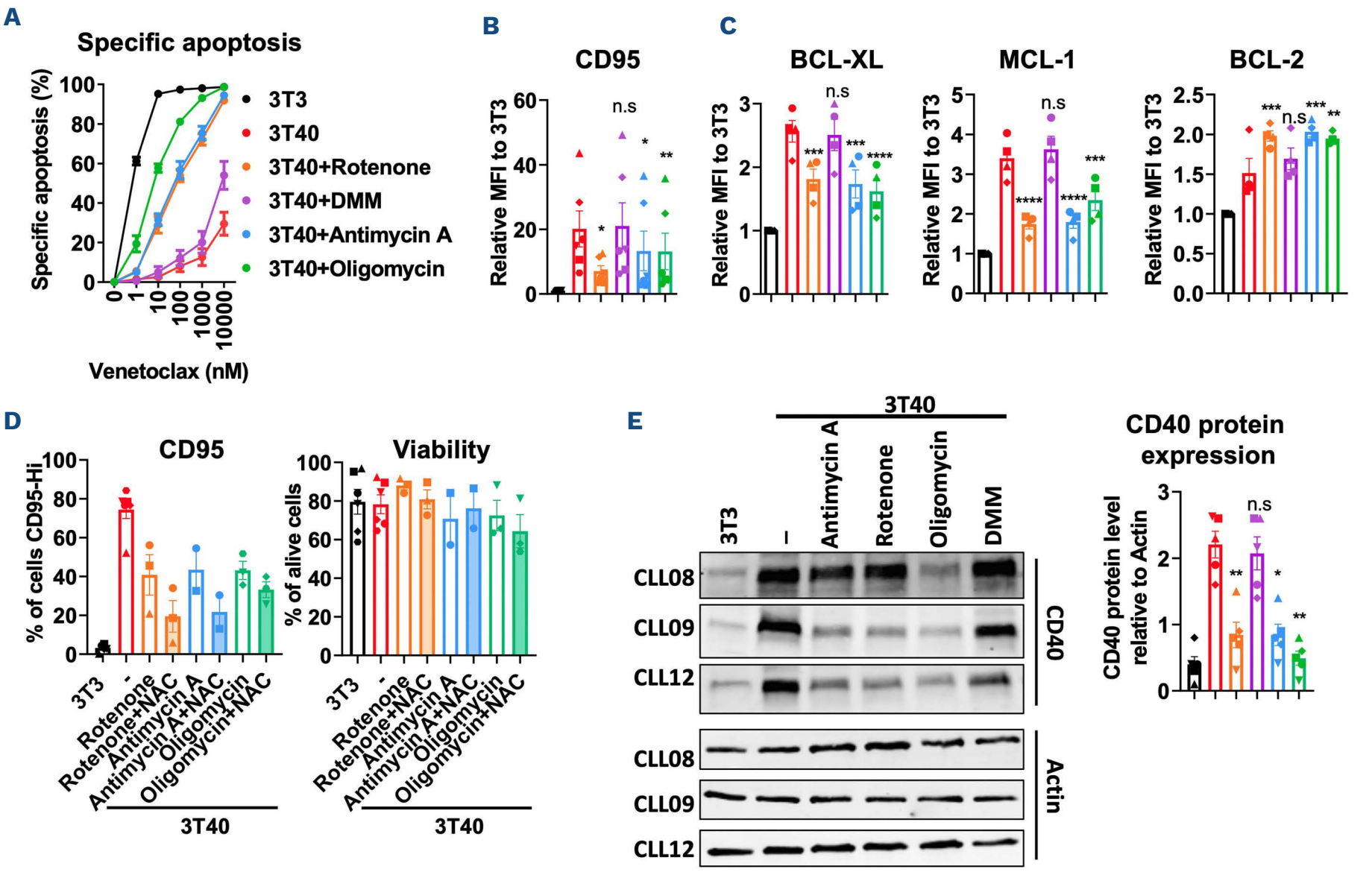

Figure 3. Inhibition of complex I, III or V of the electron transport chain during CD40 stimulation affects expression of BCL-2 members and resistance to venetoclax. Chronic lymphocytic leukemia (CLL) cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with various electron transport chain inhibitors for 24 h. (A) After resuspension, CLL cells were incubated with a titration of 1-10,000 nM venetoclax for 24 h after which viability was measured using MitoTracker Orange CMTMROS and TO-PRO-3. Specific apoptosis was calculated and is presented (N=4). (B) Right after the resuspension of CLL cells, expression of CD95 (N=6), and (C) BCL-2 family members BCL-XL (N=4), MCL-1 (N=4) and BCL-2 (N=4) were measured by flow cytometry. (D) CLL cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with rotenone (N=3), antimycin A (N=2) or oligomycin (N=3) with or without N-acetyl-L-cysteine for 24 h. After resuspension, CD95 and viability were measured by flow cytometry. (E) CLL cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with various electron transport chain inhibitors for 24 h. After resuspension, CD40 protein expression was measured by western blot; a representative scanning of three independent experiments is shown. The bar chart shows the relative quantification of CD40 expression to actin (N=5). (F) CD40 expression at the RNA level was measured by quantitative polymerase chain reaction (N=4). Data are shown as mean ± standard error of mean. One-way analysis of variance was performed to compare growth on 3T40 fibroblasts to the other conditions. Each patient is labeled with a distinct symbol across all the conditions. **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001; n.s.: not significant. DMM: dimethyl malonate; MFI: mean fluorescence intensity; NAC: N-acetyl-L-cysteine.

fore, we measured newly translated proteins after treatment with oligomycin, AZD8055 or rapamycin during CD40 activation to determine this effect. After CD40 activation, CLL cells generated much more newly synthesized proteins and, indeed, rapamycin and AZD8055 but also oligomycin counteracted this increase (Figure 6A). Broadly, this shows that these agents do not seem to affect the CD40-mediated activation of the CLL cells, but more specifically the consequences on protein synthesis.

As ETC and mTOR inhibition showed overlapping effects, we determined whether these can act complementarily by performing synergy tests. Combinations of various concentrations of oligomycin and AZD8055 or rapamycin were added to CLL cells during CD40 activation. The results showed that inhibitors by themselves had no effect on cell viability, and that only at high doses did the combinations decrease cell viability (*Online Supplementary Figure S6A*). Oligomycin and AZD8055 exhibited dosage-dependent suppression of CD95 expression, and effects of combinations on CD95 were comparable to those of oligomycin alone (*Online Supplementary Figure S6B*). Strikingly, a synergistic effect was clear in venetoclax sensitivity tests; oligomycin with AZD8055 or rapamycin sensitized CLL cells to venetoclax much more than the inhibitors alone. This was tested with various combinations of mTOR inhibitors, oligomycin and venetoclax (Figure 6B, *Online Supplementary Figure S6C*), which were able to almost fully revert the treated cells to the venetoclax sensitivity of control cells. In line with the stronger effect of AZD8055 as a single agent, synergy of AZD8055 with oligomycin was stronger than that of oligomycin with rapamycin, as shown by the synergy score tables (Figure 6C). In conclusion, collectively these data indicate that suppression of protein translation is a common effect of both ETC and mTOR inhibition, and dual inhibition acts synergistically to converge on an almost complete reversal of CD40-mediated resistance to venetoclax.

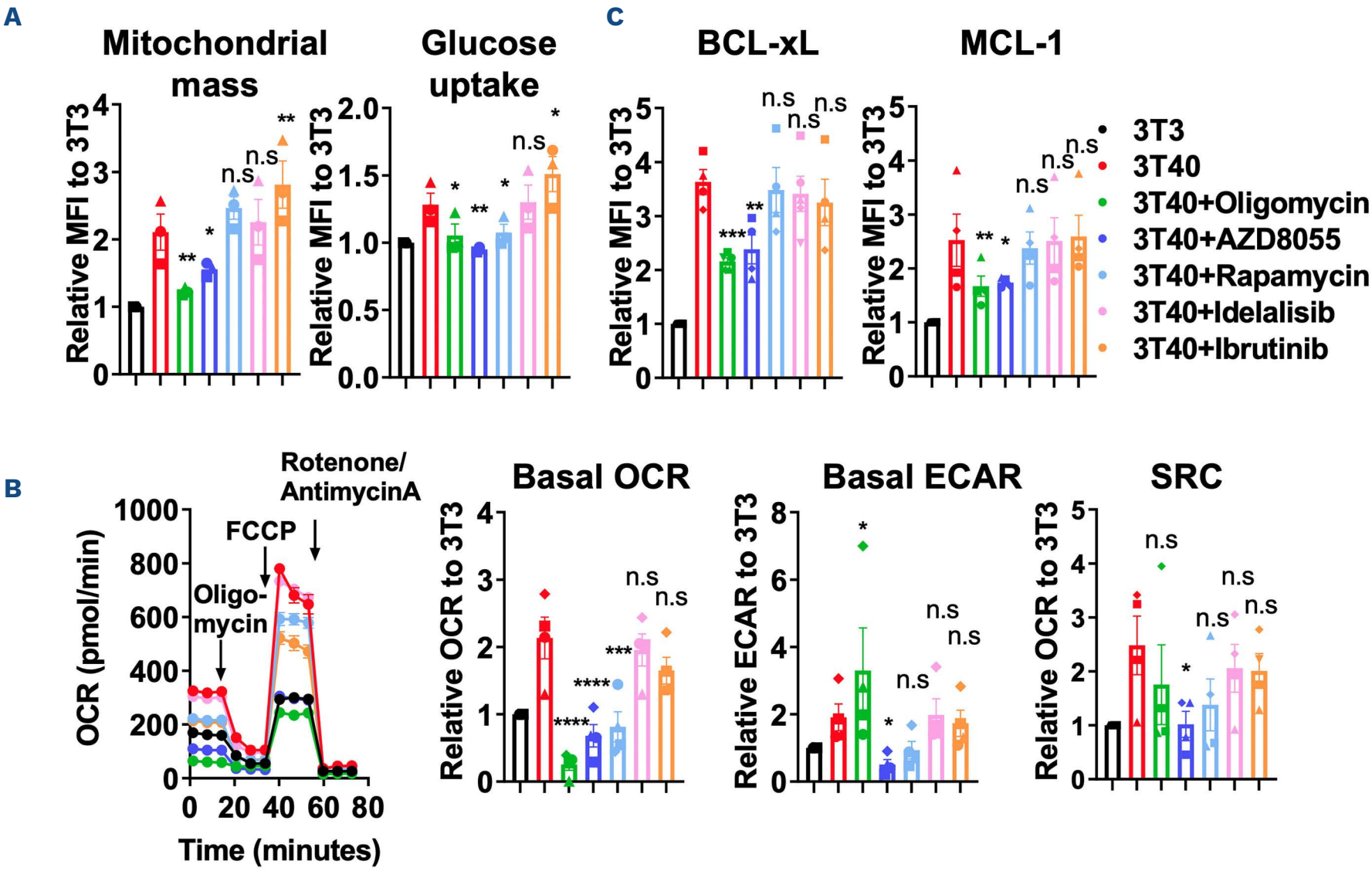

Figure 4. mTOR1/2 inhibition suppresses metabolic activities and expression of BCL-XL and MCL-1. Chronic lymphocytic leukemia (CLL) cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with various inhibitors of downstream regulators of CD40 signaling for 24 h. After resuspension, (A) mitochondrial mass (N=3) and glucose uptake (N=3) were measured by flow cytometry. (B) A Seahorse MitoStress test was performed after CLL cells were collected (N=4), basal oxygen consumption rate, basal extracellular acidification rate and spare respiration capacity were measured and calculated. (C) Intracellular staining of BCL-xL (N=4) and MCL-1 (N=4) was done after resuspension of CLL cells and their expressions were measured by flow cytometry. Data are shown as mean ± standard error of mean. One-way analysis of variance was performed to compare culture on 3T40 fibroblasts to the other conditions. Each patient is labeled with a distinct symbol across all the conditions. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; n.s.: not significant. MFI: mean fluorescence intensity; OCR: oxygen consumption rate; FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; ECAR: extracellular acidification rate; SCR: spare respiration capacity.

Discussion

The mitochondrion is crucial for both energy production and survival/apoptosis sensing. In this study, the contribution of metabolic pathways to CD40-induced venetoclax resistance was examined. By attenuating the expression of the anti-apoptotic proteins BCL-XL and MCL-1, which are localized on mitochondria where OXPHOS is also taking place, ETC inhibition clearly exhibits the capacity to counteract venetoclax resistance. In contrast, inhibition of the utilization of glucose, glutamine or long-chain fatty acids in the TCA cycle did not attenuate venetoclax resistance. This might indicate that other fuels can be used by activated CLL cells and that the mechanisms of venetoclax resistance are linked to maintenance of the ETC.

Recently, we showed that upon CD40 stimulation, CLL cells upregulate a wide range of metabolic pathways, many of them related to mitochondrial metabolism, and that glutamine contributes more than glucose to the TCA cycle in CD40-stimulated cells.[24] In the present study we have extended this by showing that only inhibition of the conversion of glutamine to glutamate, but not inhibition of pyruvate utilization in the mitochondria, decreased oxygen consumption rate. Interestingly, when using an inhibitor of the plasma membrane glutamine transporter ASCT2, CLL cells became more sensitive to venetoclax,[24] while only inhibiting glutaminolysis did not have this effect. This can be explained by the essential contribution of glutamine to other biosynthetic pathways such as nucleotide synthesis or tRNA loading for protein synthesis. In addition, ASCT2 also transports other amino acids; for

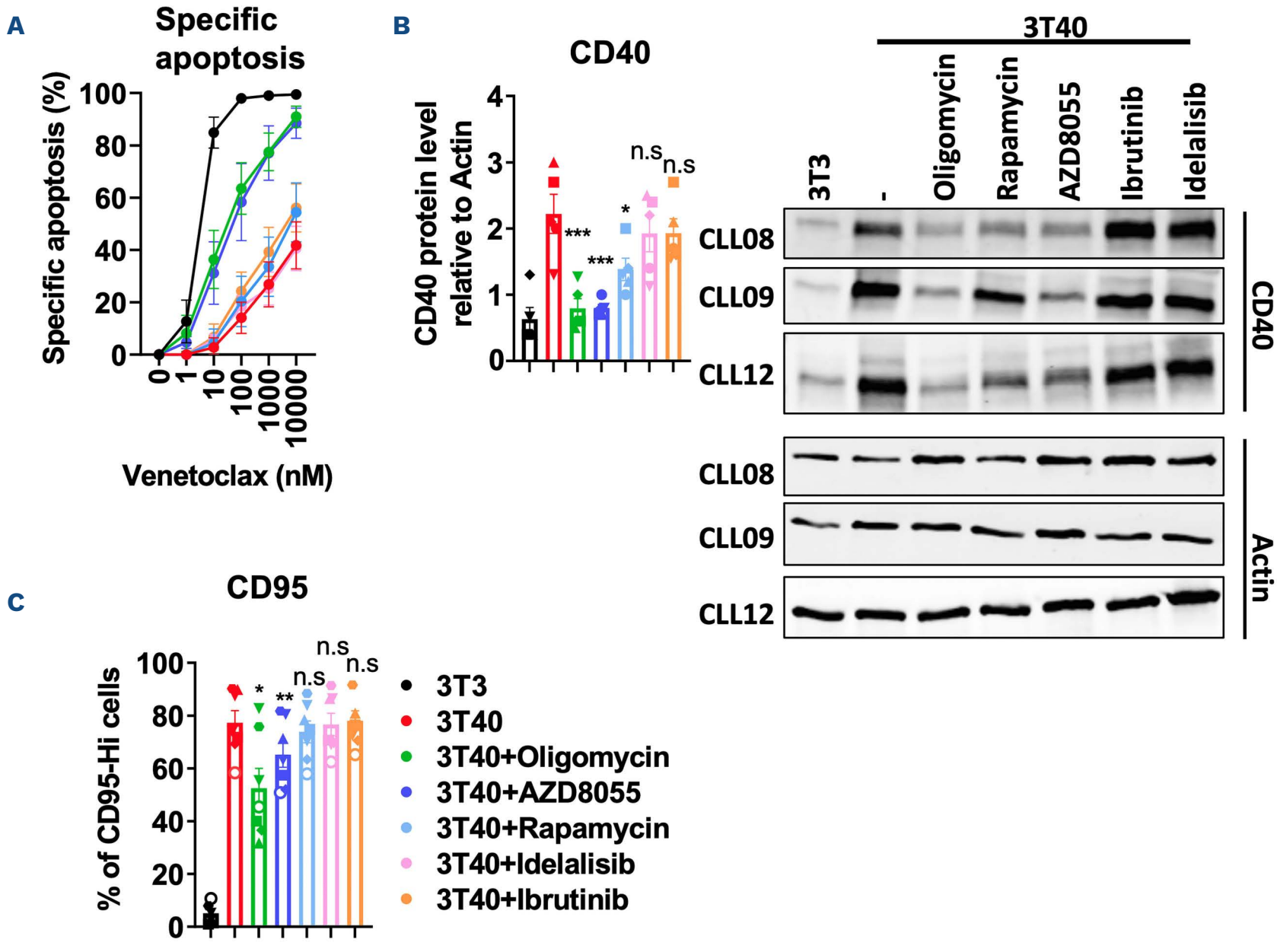

Figure 5. mTOR1/2 inhibition affects venetoclax sensitivity and CD40 expression. Chronic lymphocytic leukemia (CLL) cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with various inhibitors of downstream regulators of CD40 signaling for 24 h. (A) After resuspension, CLL cells were incubated with a titration of 1-10,000 nM venetoclax for 24 h after which viability was measured by flow cytometry using MitoTracker Orange CMTMROS and TO-PRO-3. Then specific apoptosis was calculated (N=5) (B) After resuspension, protein of CLL cells was directly isolated and CD40 protein expression was measured by western blot. A representative scanning of three independent experiments is shown. The bar chart shows the relative quantification of CD40 expression to actin (N=5). (C) CD95 was measured by flow cytometry (N=7). Data are shown as mean ± standard error of mean. One-way analysis of variance was performed to compare growth on 3T40 fibroblasts to the other conditions. Each patient is labeled with a distinct symbol across all the conditions. *P<0.05; **P<0.01; ***P<0.001; n.s.: not significant.

example, alanine metabolism was also a hit in our previous metabolomics and pathway analyses.[24]

We have identified key aspects of how the ETC interacts with CD40 signaling to affect venetoclax resistance, most prominently by attenuating the requisite protein synthesis of CD40 itself, and pro-survival BCL-2 proteins MCL-1 and BCL-XL. Various searches into clinical venetoclax resistance did not reveal a unifying underlying mechanism, although

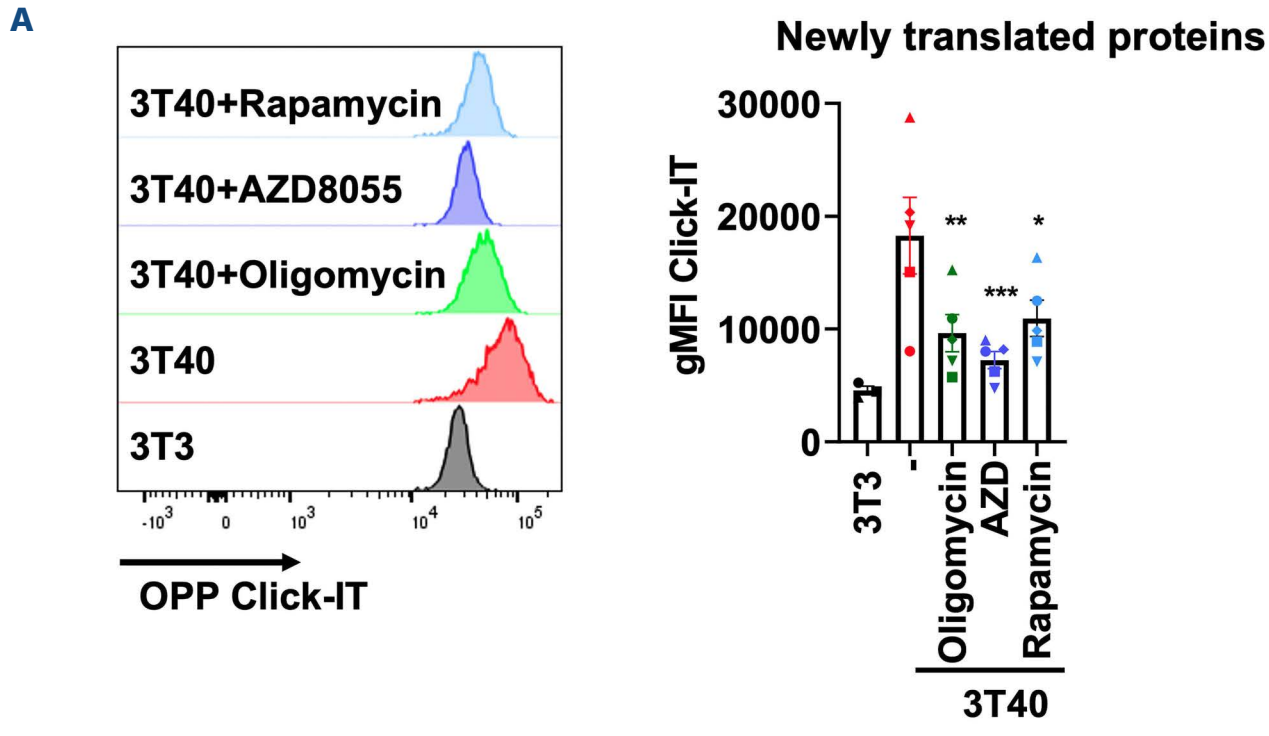

Figure 6. Cellular translation is suppressed by electron transport chain and mTOR inhibition. (A) Chronic lymphocytic leukemia (CLL) cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with oligomycin, AZD8055 or rapamycin for 24 h. After resuspension, newly translated proteins were measured with a Click-iT Plus OPP Kit (N=4). (B) CLL cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with 5 nM oligomycin, 250 nM AZD8055, 500 nM rapamycin or combinations for 24 h. After resuspension, cells were incubated with 1-10,000 nM venetoclax for 24 h after which viability was measured by flow cytometry using MitoTracker Orange CMTMROS and TO-PRO-3. Then specific apoptosis was calculated (N=3). (C) CLL cells were cultured on 3T3 or 3T40 fibroblasts and simultaneously treated with various concentrations of oligomycin, AZD8055, rapamycin or combinations for 24 h. After resuspension, CLL cells were incubated with 10 or 100 nM venetoclax for 24 h after which viability was measured by flow cytometry using MitoTracker Orange CMTMROS and TO-PRO-3. Synergy scores for electron transport chain/mTOR inhibitors and venetoclax were calculated (from *Online Supplementary Figure S6C*) (N=3). The scores in blue represent no synergy whereas those in red indicate strong synergy. Data are shown as mean ± standard error of mean. One-way analysis of variance was performed to compare culture on 3T40 fibroblasts to the other conditions. Each patient is labeled with a distinct symbol across all the conditions. *P<0.05; **P<0.01; ***P<0.001; n.s.: not significant. MFI: mean fluorescence intensity; AZD: AZD8055; VEN: venetoclax; Oligo: oligomycin; Rapa: rapamycin.

in many instances changes in BCL-2 family members were noted, including BCL-2 mutations, induction of BCL-XL, MCL-1, and BFL-1, and deletion of Bax.[18,20,36,37] Importantly, several reports have also linked venetoclax resistance to mitochondrial metabolism, mostly focusing on the selection of resistant cell lines obtained after extended culture in the presence of venetoclax.[18,19] Furthermore, changes in metabolism, specifically higher OXPHOS, were found in samples from patients who developed venetoclax resistance.[18,20,38] Our study applied a distinct approach in which primary CLL cells were activated *in vitro* with CD40 signaling which mimics the LN-TME and rapidly induces venetoclax resistance. Intriguingly, TME-inducible venetoclax resistance exhibited a similar higher metabolic status as long-term selection under therapy or in the laboratory. This indicates that CLL cells that become resistant to venetoclax in patients due to either LN-TME signals or long-term venetoclax treatment might have comparable metabolic characteristics. This notion is further supported by the fact that ETC inhibitors attenuate venetoclax resistance in both settings, as we found in this study and as previously noted by others.[18,20]

Our data also demonstrate a unique function of mTOR1/2 signaling in sustaining venetoclax resistance. While both rapamycin and AZD8055 decreased the metabolic activity of CLL cells, only AZD8055 as a single agent sensitized the cells to venetoclax. ETC inhibitors and AZD8055 lowered all regulators linked with venetoclax sensitivity, including BCL-2 family members and CD40 activation, whereas rapamycin did not. Yet importantly, despite variations in their effects on CD40 signaling and venetoclax sensitivity, AZD8055 and rapamycin exhibited a synergy combined with ETC inhibition on venetoclax sensitivity, leading to almost complete reversal of CD40-induced venetoclax resistance. As a unifying feature general protein translation was inhibited by ETC inhibition, and rapamycin and AZD8055 had a similar effect. Of note, p-eIF2a^{S51}, a key translational regulator,[39] was upregulated by CD40 (*Online Supplementary Figure S5*). P-eIF2a^{S51} is generally known to inhibit protein synthesis, but it can also promote the selective translation of some specific mRNA that are essential for cellular adaptation to stress.[40] As we and others found that protein translation is upregulated in CLL TME,[41,42] these apparent counteracting responses may be an interesting subject for future study.

In addition to CD40, other TME signals may promote venetoclax resistance in CLL cells. Previously, we determined that B-cell receptor-induced venetoclax resistance can be counteracted by inhibiting glutamine import.[24] More recently, we reported that TLR9 can also contribute to venetoclax resistance in patients under treatment with ibrutinib.[32] The effect of TLR9 in that setting was to boost protein translation, which is the opposite of the effects of mTOR and ETC inhibition reported here.

As both mTOR and ETC inhibitors have been authorized for use in clinical trials, the synergistic impact of combining ETC and mTOR inhibition may provide an opportunity for enhancing the efficacy of venetoclax. Currently, a large number of clinical trials apply mTOR inhibitors in various cancers,[43-46] and also some clinical trials regarding ETC inhibition, are ongoing. Metformin, a putative complex I inhibitor, has been tried in combination with several therapies to treat cancers, e.g., lung adenocarcinoma, ovarian cancer and breast cancer, with limited success.[47-50] The complex I inhibitor IACS-010759 had antitumor activity in brain tumor and acute myeloid leukemia.[51,52] IACS-010759 was also studied *in vitro* on CLL cells; it inhibited OXPHOS but exhibited minimal cytotoxicity.[53] Other drugs that target mitochondrial metabolism have been approved for clinical trials. CB-839, which inhibits glutaminolysis, and CPI-613, which inhibits the TCA cycle, are in clinical trials for various types of cancer.[54-57] In addition, preclinical research indicates that targeting OXPHOS is a promising strategy for enhancing the efficacy of venetoclax in acute myeloid leukemia.[52,58,59] There is a paucity of research relating to metabolism and venetoclax, especially for activated CLL in a TME setting. Our research demonstrated the synergy of OXPHOS and mTOR inhibition in relation to venetoclax sensitization, which may hold promise for clinical applications.

Disclosures

No conflicts of interest to disclose.

Contributions

EE, GJWvdW, and ZC conceptualized the study. ZC, EE, GJWvdW, and APK were responsible for the methodology. ZC, GC, and VC performed investigations. APK was responsible for patients' samples. ZC, GC, and EE were responsible for visualization. EE and APK acquired funding. EE and ZC were project administrators. EE, APK, and GJWvdW supervised the study. ZC. EE, and GC wrote the original manuscript draft. ZC, GC, HS-M, APK, GJWvdW, and EE reviewed and edited the manuscript.

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Data-sharing statement

The original data are not shared online. The details of the experimental protocols are described in the Methods section and figure legends.

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