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ORIGINAL PAPER

Suppression of MYC by PI3K/AKT/mTOR pathway inhibition in combination with all-trans retinoic acid treatment for therapeutic gain in acute myeloid leukaemia

Sven Stengel[1] | **Kevin R. Petrie**[2] | **Yordan Sbirkov**[3,4] | **Clara Stanko**[5,6] | **Faezeh Ghazvini Zadegan**[5,6] | **Veronica Gil**[7,8] | **Rafał Skopek**[9] | **Paweł Kamiński**[10] | **Łukasz Szymański**[9] | **Annamaria Brioli**[11] | **Arthur Zelent**[9] | **Tino Schenk**[5,6]

[1]Division of Gastroenterology, Hepatology and Infectious Diseases, Department of Internal Medicine IV, Jena University Hospital, Jena, Germany

[2]School of Medicine, Faculty of Health Sciences and Wellbeing University of Sunderland, Sunderland, UK

[3]Medical University of Plovdiv, Plovdiv, Bulgaria

[4]Research Institute at Medical University of Plovdiv, Plovdiv, Bulgaria

[5]Department of Hematology and Medical Oncology, Clinic of Internal Medicine II, Jena University Hospital, Jena, Germany

[6]Institute of Molecular Cell Biology, CMB, Jena University Hospital, Jena, Germany

[7]The Institute of Cancer Research, London, UK

[8]The Royal Marsden NHS Foundation Trust, Sutton, UK

[9]Department of Molecular Biology, Institute of Genetics and Animal Biotechnology, Polish Academy of Science, Magdalenka, Poland

[10]Department of Gynecology and Oncological Gynecology, Military Institute of Medicine, Warsaw, Poland

[11]Clinic of Internal Medicine C, Hematology and Oncology, Stem Cell Transplantation and Palliative Care, Greifswald University Medicine, Greifswald, Germany

Correspondence
Sven Stengel, Division of Gastroenterology, Hepatology and Infectious Diseases, Department of Internal Medicine IV, Jena University Hospital, Jena, Germany.
Email: sven.stengel@med.uni-jena.de

Tino Schenk, Department of Hematology and Medical Oncology, Jena University Hospital, Jena, Germany.
Email: tino.schenk@med.uni-jena.de

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Summary

Aberrant activity of the phosphatidylinositol-3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR [PAM]) pathway, as well as suppressed retinoic acid signalling, contribute to enhanced proliferation and the differentiation blockade of immature myeloid cells in acute myeloid leukaemia (AML). Inhibition of the PAM pathway was shown to affect especially mixed-lineage leukaemia-rearranged AML. Here, we sought to test a combined strategy using small molecule inhibitors against members of the PAM signalling pathway in conjunction with all-trans retinoic acid (ATRA) to target a larger group of different AML subtypes. We find that ATRA treatment in combination with inhibition of PI3K (ZSTK474), mTOR (WYE132) or PI3K/mTOR (BEZ235, dactolisib) drastically reduces protein levels of the proto-oncogene MYC. In combination with BEZ235, ATRA treatment led to almost complete eradication of cellular MYC, G1 arrest, loss of clonal capacity and terminal granulocytic differentiation. We demonstrate that PAM inhibitor/ATRA treatment targets MYC via independent mechanisms. While inhibition of the PAM pathway causes MYC phosphorylation at threonine 58 via glycogen synthase

Arthur Zelent and Tino Schenk these authors contributed equally to this work.

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kinase 3 beta and subsequent degradation, ATRA reduces its expression. Here, we present an approach using a combination of known drugs to synergistically reduce aberrant MYC levels, thereby effectively blocking proliferation and enabling differentiation in various AML subtypes.

KEYWORDS

acute myeloid leukaemia, all-trans retinoic acid, BEZ235, dactolisib, leukaemia, mammalian target of rapamycin, MYC, protein kinase B (AKT), phosphatidylinositol-3 kinase/protein kinase B/mammalian target of rapamycin (PAM), phosphatidylinositol-3 kinase

INTRODUCTION

Acute myeloid leukaemia (AML) is characterised by two major hallmarks: enhanced proliferation and block in differentiation of immature myeloid cells. Both processes are tightly linked in AML and increased activity of the phosphatidylinositol-3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR [PAM]) signalling pathway has been widely implicated in their regulation.[1] PAM signalling was found to be overactive in 50%–80% of AML cases and to negatively correlate with disease-free and overall survival of patients with AML.[2,3] Hyperactivation of PI3K is often driven by activating mutations in FMS-like tyrosine kinase-3 (FLT3), c-KIT and RAS proto-oncogenes.[4,5] PI3K activity leads to the activation of AKT, which is mediated by phosphorylation at Thr308 by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Ser473 by mTOR complex 2 (mTORC2). Thr308 and Ser473 phosphorylation of AKT was detected in 50%–72% of patients with AML.[6,7]

One of the many downstream targets of the PAM pathway is the proto-oncogene *MYC*. *MYC* encodes a basic helix–loop–helix leucine zipper transcription factor with important roles in cellular metabolism, apoptosis, differentiation and cell cycle progression.[8–14] MYC overexpression is commonly found in AML and has been widely implicated in its initiation and maintenance.[8,9,13,14]

Among others, MYC levels are controlled by regulation of its stability.[15] Phosphorylation at threonine 58 (T^{58}) has been shown to induce proteasomal degradation of MYC. MYC T^{58} is phosphorylated by the serine/threonine-specific protein kinase glycogen synthase kinase 3 beta (GSK3B), which is a direct target of AKT.[16]

Besides controlling the stability of MYC via GSK3B, AKT is also an important mediator of the activity of mTORC1. Activation of mTORC1 leads to phosphorylation of p70 S6 kinase (p70S6K), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), which in turn increases the translation of MYC.[1,14,17]

These observations form the rational for the use of small molecule inhibitors that impair PI3K, as well as mTORC1 and mTORC2, to target MYC stability and translation in AML.

Dimberg et al.[18] showed that MYC transcription can be impaired by the vitamin A derivative all-trans-retinoic acid (ATRA) in AML blasts. ATRA has a high potential as a therapeutic agent for the treatment of haematopoietic malignancies, due to its function as an inducer of terminal differentiation of leukaemic blasts. To date, it has shown its most potent activity in acute promyelocytic leukaemia (APL), where it induces complete remission in the majority of cases when given in combination with arsenic trioxide (ATO) or chemotherapy. It has been studied in combinations with other drugs in a variety of cancers and precancerous conditions and several clinical trials using ATRA as part of combined therapies are ongoing.

Here, we sought to combine inhibitors of PAM signalling with ATRA in an attempt to induce maximal MYC suppression to reduce the proliferation and stemness of AML blasts.

MATERIALS AND METHODS

Cell lines, primary samples and cell culture

All cell lines (MV4-11, HL-60, OCI-AML2, OCI-AML3, THP-1, MOLM-13, KG1a and NB4) were obtained from the American Type Culture Collection (ATCC) and maintained according to their recommendations (www.atcc.org). Peripheral blood or bone marrow mononuclear cells from patients with AML were isolated by Ficoll density gradient separation, cultured in Stemline II Medium (Sigma, Hamburg, Germany), and treated as indicated. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki and the study was approved by the Ethics committee of the University Hospital Jena.

Myeloid differentiation, apoptosis and colony forming assays

Cells were treated with ATRA (Sigma), ZSTK474 (Selleck Chemicals), WYE132 (Selleck Chemicals) or BEZ235 (Selleck Chemicals) for 2 days prior to analysis.

Expression of the differentiation maker CD11b on MV4-11 cells was estimated by flow cytometry. At least 5×10^5 cells were stained with a phycoerythrin (PE)-conjugated human CD11b-specific mouse monoclonal antibody (BD Pharmingen, #555388) and analysed on a BD LSRII flow cytometer (Becton Dickinson) using CellQuest software. Cell death was estimated by propidium iodide (Invitrogen) incorporation. Primary patient samples

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were analysed via flow cytometry as described above for the following differentiation markers: CD11c (PE, clone: 3.9), CD11B (fluorescein isothiocyanate [FITC], clone: ICRF44), CD34 (allophycocyanin [APC], clone: 561), CD15 (PacificBlue, clone: W6d3) and CD14 (APC/cyanine 7 [Cy7], clone: M5E2) (all antibodies Biolegend). Apoptotic cells were analysed by surface staining of the apoptosis marker phosphatidylserine using FITC-labelled Annexin V (Biolegend). Colony formation of HL-60 cells was assessed in colony-forming unit assays in methylcellulose as previously described.[19]

Cell proliferation and cell cycle arrest analyses

Inhibition of proliferation was measured using the CellTiter-Glo® luminescent cell viability assay (Promega). Cells were seeded at a density of 50 000 cells/ml for 24 h, followed by drug treatment with the indicated concentrations. Cell cycle status of cell populations was evaluated by flow cytometric analysis for propidium iodide (Invitrogen) incorporation, on a BD LSRII flow cytometer (Becton Dickinson) using CellQuest software.

Cell lysis and immunoblotting

Cell lysates were obtained using Cell Lysis Buffer (Cell Signaling Technology) in presence of Complete Protease Inhibitor Cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were separated and stained according to standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting protocols as previously described.[20] The antibodies used were as follows: anti-β-tubulin (Sigma, #SAB3500023), anti-mouse-horseradish peroxidase (HRP, Amersham, #NA9310), anti-rabbit-HRP (Amersham, # NA934-1Ml), anti-MYC (Epitomics, 1472–1), anti-PhosphoT^{58}-MYC (Abcam, ab28842), anti-AKT (#9272, #4058), anti-GSK3B (#9323, #9315), anti-RPS6 (#2317, #2211), anti-4E-BP1 (#9644, #9459) anti-β-Actin (#3700), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #2118) (all Cell Signaling Technology).

Quantitative real-time polymerase chain reaction (qPCR)

Following treatment, cells were washed in phosphate-buffered saline (PBS) and RNA was isolated using the ZR RNA MiniPrep Kit (Zymo Research). RNA concentration and quality were confirmed by spectrophotometry (NanoDrop, Thermo Scientific). Synthesis of complementary DNA (cDNA) was performed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisherScientific) and random hexamer primers as described by the manufacturer. The following primer pairs were used for qPCR: GUSB-FW: AAACGATTGCAGGGTTTCACC, GUSB-REV: GCGTTTTTGATCCAGACCCA, MYC-FW: CACCACC AGCAGCGACTC, MYC-REV: TGTGAGGAGGTTTGC TGTGG. Samples were analysed in triplicate utilising the MESA FAST qPCR Mastermix Plus for SYBR Green (Eurogentec) on a 7900 Fast Real Time PCR System (Applied Biosystems) as previously described.[21] Melting curves were generated for quality control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold change.[22]

Affymetrix gene expression analysis

Cells were treated with 0.1 μM ATRA, 0.1 μM BEZ235 or with both agents simultaneously for 3, 12 or 24 h. Then, cells were washed in PBS and total RNA was isolated as described above. We assessed the concentration and quality of the total RNA by spectrophotometry (NanoDrop, Thermo Scientific) and by using an Agilent 2100 BioAnalyzer (Agilent Technologies). Global gene expression profiling was conducted using Affymetrix GeneChip® Human Gene 1.0 ST Arrays and corresponding chemistry. Briefly, second cycle single-stranded cDNA synthesis was carried out using the GeneChip® WT PLUS Reagent Kit in combination with the GeneChip® Eukaryotic Poly-A RNA Controls and 200 ng of total RNA. Then, sc-cDNA was fragmented and labelled using the GeneChip WT Terminal Labelling Kit. Then, array-hybridisation, -washing and -staining was performed using GeneChip Expression Hybridisation Controls and the GeneTitan® Hybridisation, Wash, and Stain Kit for WT Array Plates. Hybridisation was performed in a GeneChip Hybridisation Oven for 16 h at 45°C with rotation at 60 rpm. The wash and stain was performed on a GeneChip Fluidics Station 450. Arrays were scanned on a GeneChip Scanner 3000. All reagents and equipment were purchased from Affymetrix and work was performed according to the protocols provided by the manufacturer.

Statistical analysis

Gene array data were analysed using Bioconductor version 3.11[23] and the R statistical programming language version 4.0.0 [R Development Core Team R: A language and environment for statistical computing, R foundation for statistical computing, https://www.r-project.org/: Vienna, Austria, 2010]. Thereby, robust multichip average (RMA) background correction and normalisation was done using the simpleaffy package version 2.66.0.[24] For quality control we used the packages affyPLM version 1.66.0 [doi:10.1007/0-387-29362-0_3]. Differentially expressed genes were identified using Limma package version 3.46.0[25] and annotated using the hugene10sttranscriptcluster.db package version 8.7.0 and the annotate package version 1.68.0. The enrichment analysis of differentially expressed genes was performed using Gene Set Enrichment Analysis (GSEA) tool from Broad Institute[26] using the following settings: Permutation type – gene set;

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Number of permutations – 1000; Gene Set Database – H: Hallmark gene sets from Molecular Signature Database version 6.2. Heatmaps, dot plots, Venn diagrams and principal component analysis were conducted in R. The data were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, if not stated otherwise. Values with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism software (version 9; GraphPad Software Inc.).

RESULTS

Combined treatment with PAM inhibitors and ATRA leads to G1 arrest, differentiation and apoptosis

To evaluate the combinatorial effects of PAM inhibition (PAMi) and ATRA treatment on AML cell proliferation and differentiation, MV4-11 cells were treated with different concentrations of the PI3K inhibitor ZSTK474, the mTOR inhibitor WYE132 and the dual PI3K/mTOR inhibitor BEZ235 alone and in combination with ATRA. Single treatment with low nanomolar concentrations of ZSTK474 and WYE132 had only minor inhibitory effects on cell growth but 10 nM BEZ235 reduced cell growth by 63% relative to the untreated population. Single ATRA treatment led to a reduction by 34%. The combination of ATRA with WYE132 or ZSTK474 drastically decreased, and the combination with BEZ235 fully abrogated proliferation (Figure 1A; Figures S1A and S8). Cell cycle analysis showed a dose-dependent increase in the percentage of cells in G1 after treatment with single compounds of which BEZ235 was most potent. Co-treatment with ATRA further increased the percentage of cells in G1 arrest to >90% (Figure 1B; Figure S1B). Induction of myeloid differentiation was assessed measuring CD11b surface levels. Combined treatment with ATRA and BEZ235 synergistically increased the percentage of cells expressing CD11b and induced subsequent apoptosis (Figures 1C and 3C). ZSTK474 and WYE132 showed no effect on CD11b expression (Figure S1C).

Synergistic induction of gene sets important for immunological and metabolic functions, proliferation and differentiation

To examine the effects of ATRA and BEZ235 on the transcriptome of MV4-11 cells, we analysed genome-wide changes in mRNA expression following 3, 12 and 24 h treatment (Gene Expression Omnibus [GEO] accession code GSE160964; Table S1). Hierarchical clustering and principal component analyses of differentially expressed genes showed that ATRA and BEZ235 treatment initiate very distinct expression profiles, with BEZ235 having the larger impact on genome-wide gene regulation (Figure 1D; Figure S2A). However, despite the induction of distinct gene expression profiles, gene set enrichment analysis revealed that both treatments result in the regulation of largely the same cellular functions, such as the activation of P53-signalling- and the inhibition of G2M-checkpoint associated genes (Figure 1E; Figure S2B). Remarkably, the effects of the individual treatments were not only combined but potentiated by co-administration of ATRA and BEZ235 as indicated by the regulation of a large set of genes not regulated by single treatments (Figure 1F). Cellular functions of these synergistically regulated genes mostly overlapped with the functions found for single treatments (Figures S2B,C), indicating a strengthening of these anti-leukaemic pathways. In addition, combination treatment increased the early expression of selected retinoic acid receptor (RAR) targets such as cytochrome P450 family 26 subfamily A member 1 (CYP26A1), homeobox A4 (HOXA4), HOXA5, HOXA7, CCAAT/enhancer-binding protein epsilon (CEBPE) and CD38 (Table S1).

BEZ235 and ATRA synergistically inhibit MYC expression and strongly impact MYC target gene expression

The gene set with the highest enrichment score was 'MYC_TARGETS_V2' (M5928). M5928 was found in the fraction of repressed genes, pointing towards a reduction of MYC activity after treatment. In line with that, a highly significant emphasised reduction of direct MYC target genes was found for the double treatment (Figure S2D,E). Of the MYC family members MYC, MYCN and MYCL, only MYC was found to be regulated following treatments and exhibited high mRNA expression levels (Table S1), indicating that MYC rather than MYCN or MYCL is biologically active in MV4-11 cells. Expression analysis by qPCR showed a synergistic decrease in MYC mRNA levels already at 1 h and maximal reduction 4 h after treatment (Figure 1G).

Pharmacological inhibition of PAM signalling combined with ATRA treatment leads to a strong reduction of MYC protein levels in AML cells

Finding a strong reduction of MYC mRNA after PAMi/ATRA treatment, we next evaluated MYC protein levels. Immunoblotting in MV4-11 and other AML cell lines 3 h after single-agent and combination treatments revealed that all PAM inhibitors reduced the protein level of MYC with BEZ235 being most potent (Figure 2A; Figure S3A). Further reduction in MYC protein was achieved when PAMi were combined with ATRA. MYC was almost eradicated by a combined treatment with 1 μM BEZ235 and 0.1 μM ATRA. MYC depletion was accompanied by phosphorylation of its threonine at position 58 (T^{58}), an obligatory signal for MYC degradation (Figure 2A; Figure S3A).

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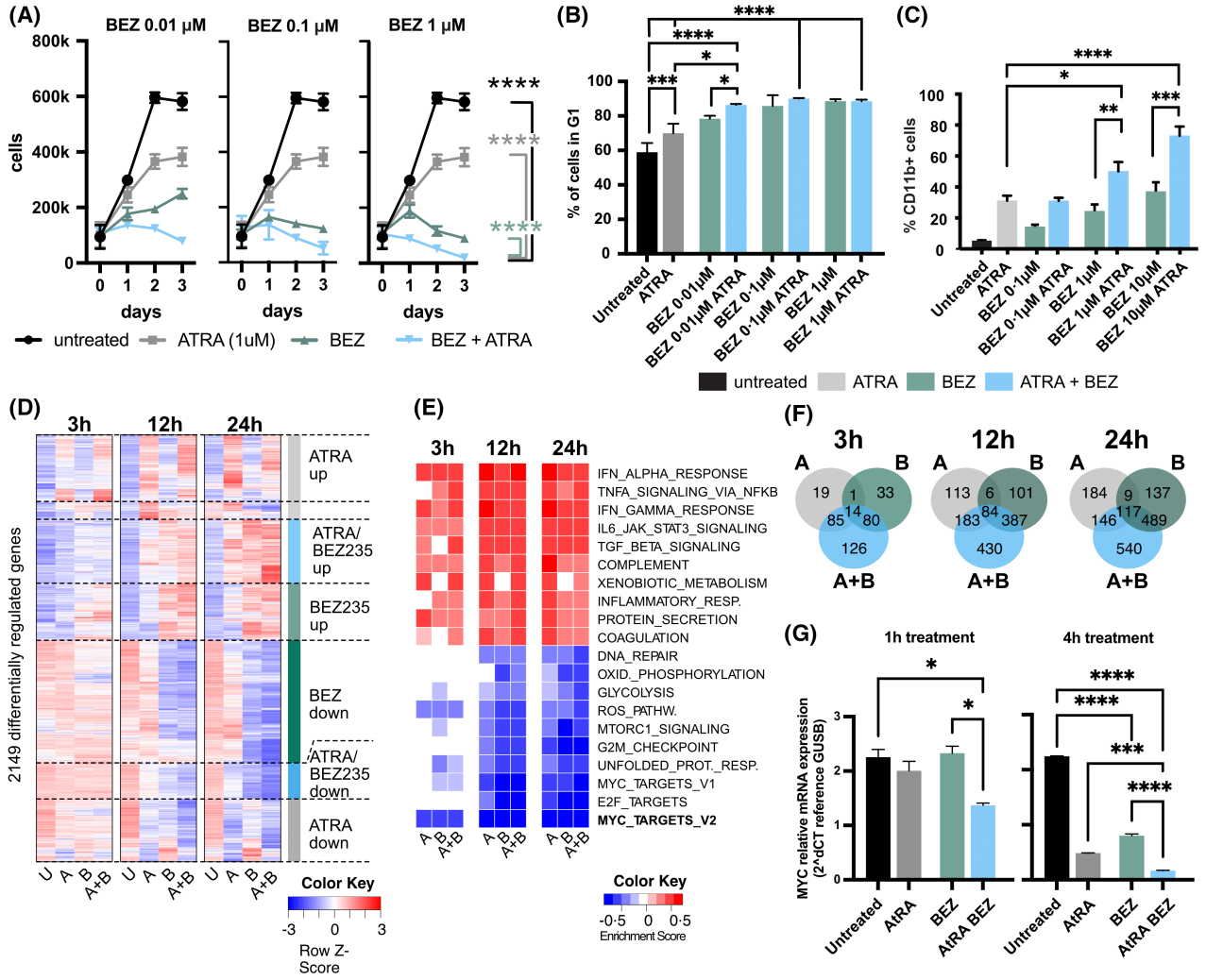

FIGURE 1 Combined treatment with phosphatidylinositol-3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) inhibitors and all-trans retinoic acid (ATRA) shows anti-leukaemic effects in MV4-11 acute myeloid leukaemia (AML) cells and leads to MYC inactivation. MV4-11 cells were treated as indicated with the dual PI3K and mTOR complex 1/2 (mTORC1/2) inhibitor BEZ235 and ATRA. (**A**) Growth of MV4-11 cells after treatment was measured for 3 days using the CellTiter-Glo® luminescent cell viability assay (Promega). (**B**) Cell cycle analysis was performed at 24 h after treatment of MV4-11 cells with the indicated concentrations of BEZ235 and 0.1 µM ATRA. Analysis was performed after cell fixation and DNA staining with propidium iodide followed by fluorescence-activated cell sorting (FACS) analysis. (**C**) Expression of the myeloid differentiation marker CD11b was analysed by FACS as indicated using a phycoerythrin-conjugated human CD11b-specific mouse monoclonal antibody. FACS analysis was performed 3 days after induction of cell differentiation. (**D**) MV4-11 cells were treated with 0.1 µM ATRA, 0.1 µM BEZ235, 0.1 µM ATRA together with 0.1 µM BEZ235 or left untreated for 3, 12 and 24 h. RNA was extracted and genome wide gene expression was investigated by microarray analysis. Differentially expressed genes were determined in comparison to untreated control cells from respective time points (treatment vs. control) and gene expression values of genes with a corrected $p < 0.01$ were plotted as a heatmap following hierarchical clustering. (**E**) Gene set enrichment analysis was performed for all treatment regimens and time points in comparison to untreated control cells using the GSEA tool (4.0.3) from UC San Diego and Broad institute and the HallmarksAll.V.7.1 database. Enrichment scores of the Top-10 up- and down-regulated gene sets were plotted as heatmap. (**F**) Number of differentially expressed genes relative to untreated control cells (adjusted $p \leq 0.05$ and fold change [FC] ≤ -2 or FC ≥ 2) plotted as Venn diagrams. (**G**) Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of MYC gene expression relative to untreated control (t = 0h). MV4-11 cells were treated with ATRA (1 µM) and/or BEZ235 (1 µM) or left untreated. Treatment was stopped at indicated time points, total RNA was isolated, reverse transcribed and MYC specific qPCR was performed

Inhibition of GSK3B can partly revert the effects of PAM inhibition

To assess if GSK3B, the kinase responsible for MYC T^{58} phosphorylation, and its activating kinase AKT are affected by the treatment we measured their absolute and phosphorylated levels. As absolute GSK3B and AKT levels stayed unchanged, a dose-dependent reduction in phosphorylation of AKT at residue S^{473}, and to a lesser extend of GSK3B at residue S^{9} occurred in the presence of all three PAMi, consistent with a pathway effect. Additional ATRA treatment did not affect the absolute and phosphorylated levels of both kinases (Figure 2A; Figure S3A). In addition, inhibition of GSK3B by SB216763 not only restored MYC protein levels and prevented MYC T^{58} phosphorylation, but also inhibited the anti-leukaemic effects

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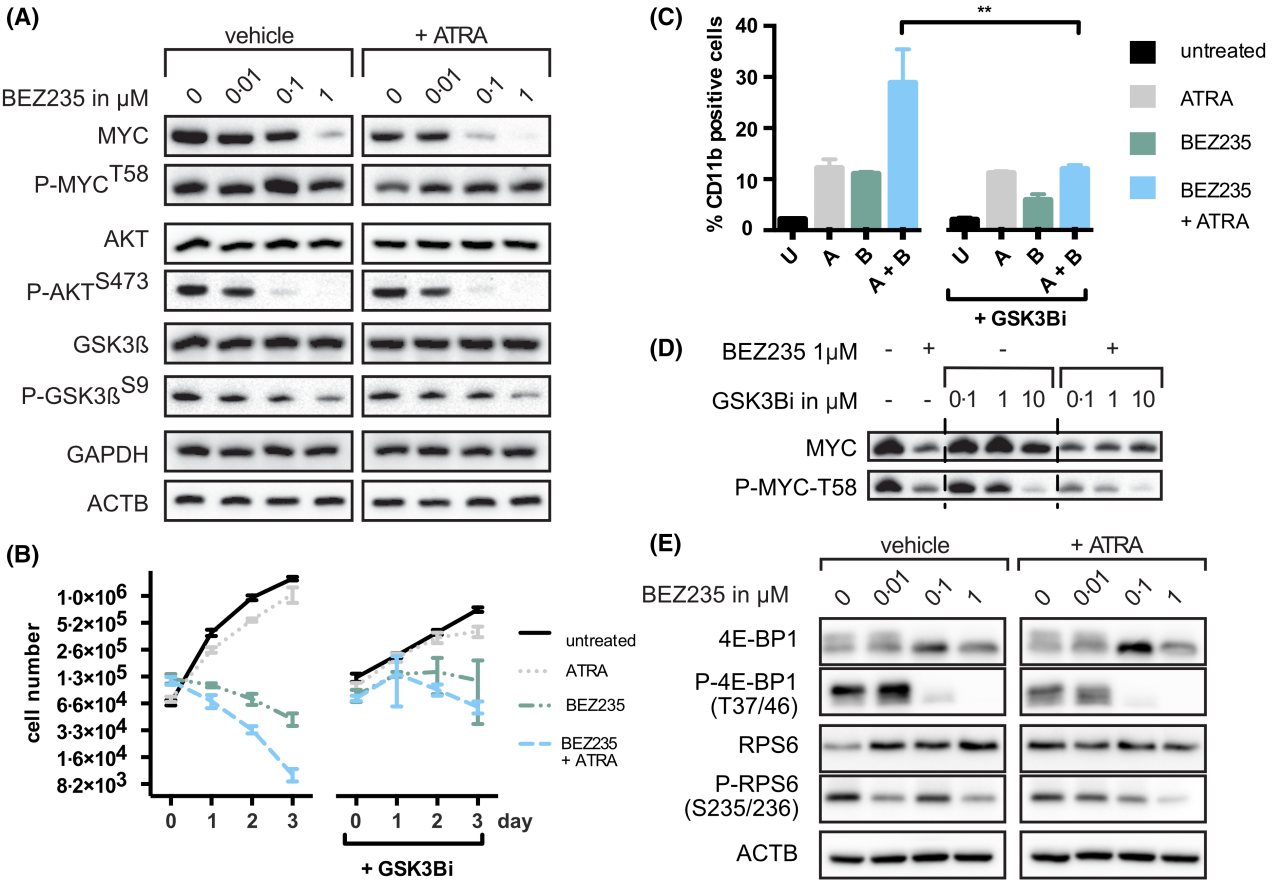

FIGURE 2 Reduction of MYC protein through (ATRA)/BEZ235 treatment and attenuation of the effect by inhibition of glycogen synthase kinase 3 beta (GSK3Bi). (**A**) MV4-11 cells were treated for 3 h with BEZ235 (1 µM), ATRA (0.1 µM) or the combination. Quantification of protein kinase B (AKT), GSK3B and MYC, as well as their phosphorylated forms (P-AKT^{S473}, P-GSK3B^{S9} and P-MYC^{T58}) was carried out by immunoblotting. (**B**) MV4-11 cells were treated with BEZ235 (1 µM), ATRA (0.1 µM) and GSK3Bi (SB216763, 10 µM). Growth of MV4-11 cells was determined by bioluminescence and effects of GSK3Bi was analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test ($p < 0.05$). (**C**) Fluorescence-activated cell sorting (FACS) expression analysis of the myeloid differentiation marker CD11b was performed 3 days after treatment as described above. Data were analysed by one-way ANOVA followed by Tukey's post hoc test. (D) Levels of phosphorylated (P-MYC^{T58}) and total MYC protein were quantified 3 h after treatment as indicated by immunoblotting. (**E**) Inhibition of protein translation pathways following BEZ235 and ATRA treatment

of BEZ235 (but not ATRA) (Figure 2B–D; Figure S4A,B). This confirms the effects of BEZ235 in GSK3B activation and MYC destabilisation.

Combined treatment with BEZ235 and ATRA reduces protein synthesis pathway activity

The mTORC1 complex is known to regulate MYC protein synthesis and cell growth via phosphorylation dependent repression of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and activation of the ribosomal protein S6 kinase (S6K) and its substrate, the ribosomal protein S6 (RPS6).[27,28] Treatment of cells with increasing concentrations of BEZ235 resulted in decreased levels of phosphorylated 4E-BP1 and RPS6, whereas the total amount of these proteins stayed relatively stable. Treatment of cells with ATRA alone led to a small reduction of 4E-BP1 and RPS6 phosphorylation when compared to untreated control cells and an enhanced reduction was found when cells were co-treated with BEZ235 and ATRA (see Figure 2E; Figure S3B). This data shows that BEZ235 and ATRA jointly attenuate the mTORC1 dependent protein synthesis pathway for a combined reduction of MYC protein levels.

The BEZ235/ATRA combination treatment is effective in different AML subtypes

After demonstrating the efficacy of the ATRA/BEZ235 combination in the MV4-11 cell, we sought to evaluate if the treatment is effective in AML with different genetic and cytogenetic backgrounds. We therefore treated eight AML cell lines and eight primary AML samples. Most cell lines responded with increased expression of CD11b, induction of apoptosis and reduced growth towards PAM inhibition by BEZ235 (Figure 3A–C). In primary AML samples ATRA/BEZ235 treatment increased

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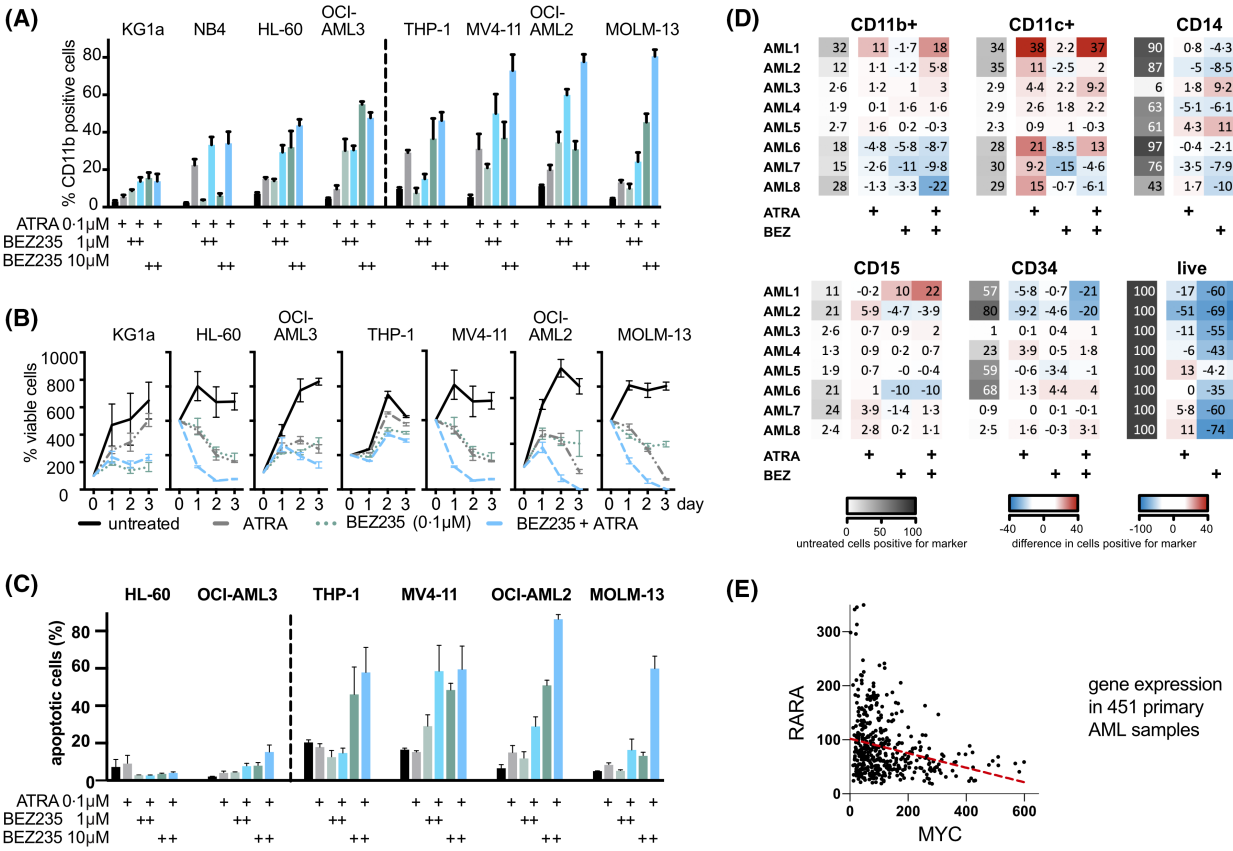

FIGURE 3 Phosphatidylinositol-3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) inhibitors in combination with all-trans retinoic acid (ATRA) reduces cell growth and induces differentiation and cell death in acute myeloid leukaemia (AML) cell lines and primary samples. AML cell lines and primary samples were treated as indicated with BEZ235 and ATRA (0.1 μM). (**A**) Surface expression of the myeloid differentiation marker CD11b was analysed by flow cytometry using a phycoerythrin-conjugated human CD11b-specific antibody in eight AML cell lines 3 days after treatment. (**B**) Growth of AML cell lines after treatment was measured for 3 days using the CellTiter-Glo® luminescent cell viability assay (Promega). (**C**) Flow cytometric analysis estimating early apoptosis by measurement of the redistribution of phosphatidylserine using annexin V staining following 3 days treatment. (**D**) Changes in differentiation marker expression in primary AML patient samples after treatment. The left rows in each panel (grey) show the percentage of cells carrying the indicated surface marker in the untreated cell population. Beside a heatmap (red-blue) of the percentages of cells that change expression following treatment are shown. The final percentage of cells carrying the indicated surface marker (not shown) would be the sum of the value in the left (grey) and side (red-blue) row. The last panel shows the change in the percentage of live cell numbers relative to the untreated sample. (**E**) Negative correlation between MYC and retinoic acid receptor alpha (RARA) expression (CPM) in 451 primary AML samples (Pearson's r = −0.254)[50]

the expression of CD11b, CD11c, CD14 or CD15 in four of eight, decreased expression of CD34 in two of eight and reduced cell viability in all samples (Figure 3D; Figure S7). Combination index calculations indicate additive or synergistic effects, depending on the applied drug concentrations (Figure S6). In contrast to Sandhöfer et al.[29] we did not observe higher sensitivity in mixed-lineage leukaemia (MLL)-translocated AML towards single treatment with BEZ235.[29] Nevertheless, the ATRA/BEZ235 combination was effective in three of four MLL-translocated AML. Immunoblotting showed that ATRA or BEZ235 alone, and even more so in combination, decreased MYC protein levels in most cell lines, broadly correlating with reduction of cell growth and differentiation (Figure S5). Supporting database analyses showed a negative correlation between MYC and RARA expression in 451 primary AML samples (Figure 3E). Samples with high RARA mRNA levels express MYC at low levels, further indicating retinoic acid receptor activity as a major suppressor of MYC expression in AML.

DISCUSSION

The PAM pathway plays a central role in the regulation of metabolism, growth, division, and survival; mechanisms often altered in tumour cells, increasing their fitness and pathogenicity.[30,31] PAM signalling is hyperactive in up to 80% of human tumours[32] including AML.[31,33] It therefore represents a promising target for treatment. However, inhibition of individual enzymes of the signalling pathway, such as mTORC1 via rapamycin showed little clinical benefit.[34,35] The use of dual mTORC1/2-PI3K inhibitors has been shown to increase anti-leukaemic effects,[27,29,36] but was associated

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with low drug tolerance in clinical trials.[37] One way to overcome this is to reduce their doses by combining them with agents that enhance their effectiveness.

Here we find that treatment of AML with very different genetic backgrounds with PAM inhibitors in combination with ATRA drastically increases anti-leukaemic effects and that this is due to a joint reduction of MYC activity (see Figure 4 for an overview).

To test the functions of different compartments of the PAM pathway in conjunction with ATRA the dual mTORC1/2-PI3K inhibitor BEZ235, the mTORC1/2 inhibitor WYE132, and the PI3K inhibitor ZSTK474 were tested. Inhibition of mTORC1/2 or PI3K by WYE132 or ZSTK474 respectively, was found to primarily induce cell cycle arrest, whereas co-inhibition of mTORC1/2 and PI3K by BEZ235 also induced differentiation, apoptosis and cell death (Figure S1). Direct comparison of different inhibitors is challenging, but inhibitor concentrations used in this study showed similar effects on PAM pathway inhibition (see activation markers P-AKT-S473, P-GSK3B-S9, P-RPS6-S235/236 in Figure S3). None the less, BEZ235 was clearly superior to WYE132 and ZSTK474 in terms of inducing anti-leukaemic effects even at lower inhibitor concentrations. This suggests that comprehensive and consistent inhibition of the PAM signalling pathway by silencing multiple master regulators, not least through the concomitant silencing of multiple negative feedback loops,[28] provides a distinct advantage for the induction of anti-leukaemic effects, such as activation of differentiation and apoptosis.

To gain more insights into the regulatory events induced by ATRA and BEZ235 co-treatment we analysed genome wide expression changes following single and double treatment of AML cells. Despite different regulation patterns on the single gene level, we identified many co-regulated cellular functions by gene set enrichment analysis. A common activation of immunological gene sets such as interferon alpha, tumour necrosis factor alpha and interleukin 6 signalling pathways confirms the activation of differentiation processes by both agents. The downregulation of glycolysis, mTORC1 signalling and cell cycle relevant genes on the other hand indicates common anti-leukaemic effects of ATRA and PAMi found also at the transcriptional level.

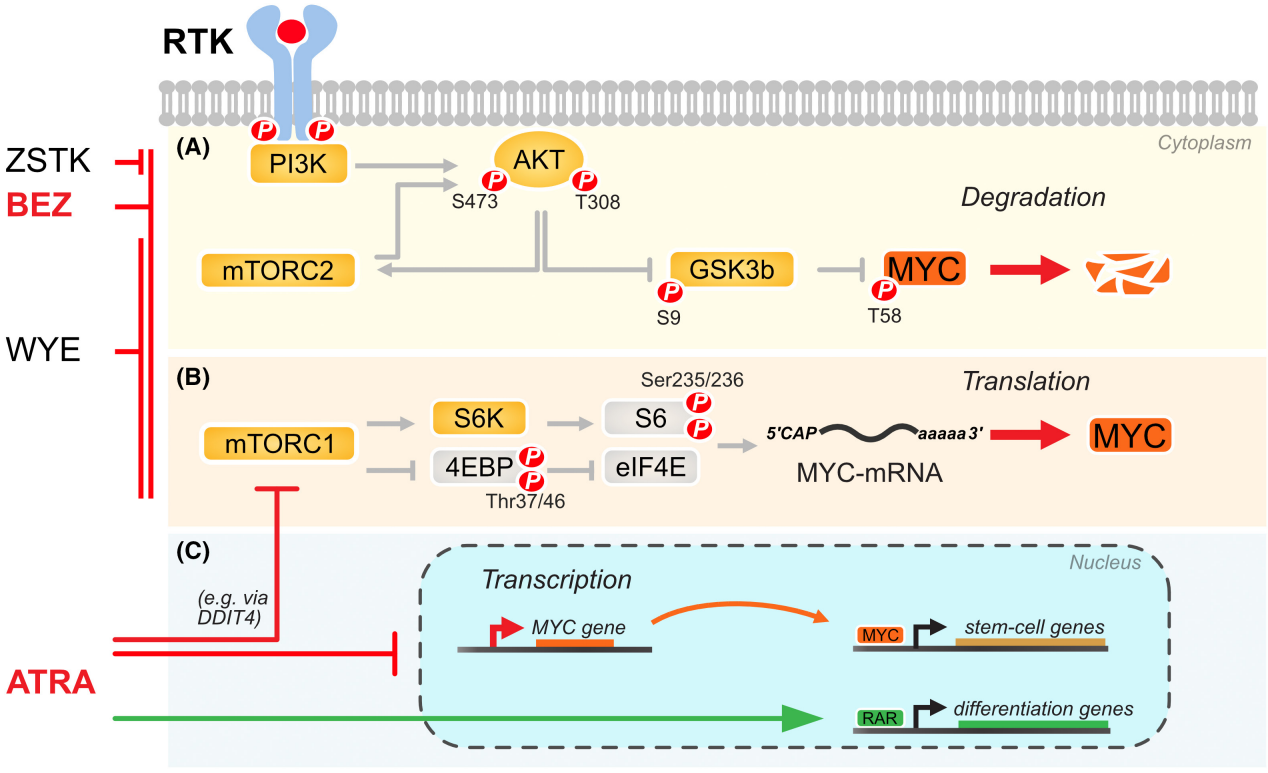

FIGURE 4 Schematic overview of MYC-targeting by phosphatidylinositol-3 kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR [PAM]) inhibitors (PAMi) and all-trans retinoic acid (ATRA). Combined treatment of acute myeloid leukaemia (AML) cells with PAMi and ATRA leads to MYC eradication via MYC degradation, translational and transcriptional inhibition. (**A**) MYC destabilisation: Treatment of leukaemic cells with the PI3K inhibitor ZSTK474 (ZSTK), the mTOR inhibitor WYE132 (WYE) or the dual PI3K/mTOR inhibitor BEZ235 (BEZ) leads to inactivation of PI3K and/or the mTOR complex 1/2 (mTORC1/2) complex and its downstream target AKT. Inhibition of AKT kinase activity in turn, causes the stabilisation of glycogen synthase kinase 3 beta (GSK3B) kinase, enhanced MYC T58 phosphorylation, and thus increased proteasomal degradation of MYC. (**B**) MYC translation: inhibition of mTORC1 as well as ATRA treatment results in 4E-BP1 mediated repression of eIF4E and the repression of the ribosomal protein S6 kinase (S6K) and its substrate, the ribosomal protein S6 (RPS6). By this, translation efficiency of mRNAs with 7-methyl-GTP cap structure and long structured 5′ untranslated regions, such as MYC mRNA is decreased. (**C**) MYC transcription: MYC was found to be a direct response gene of ATRA treatment, containing a retinoic acid receptor responsive element within its P2 promoter leading to transcriptional repression of MYC.[38] ATRA treatment was also found to inhibit mTORC1 activity via the upregulation of the early retinoic response gene DNA damage inducible transcript 4 (DDIT4), an inhibitor of mTORC1[39]

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Most strikingly, single and combined treatments were found to regulate many genes associated with the regulation of MYC targets. Most were significantly downregulated. This suggests a common effect on MYC activity. Indeed, MYC has been described as a target of ATRA as well as PAMi before.[40–42] In human monoblastic U-937 cells it has been demonstrated that ATRA directly downregulates MYC via a retinoic acid responsive element within the P2 promoter of the MYC gene.[38] We also found MYC to be transcriptionally downregulated already 1 h after ATRA treatment, indicating a direct regulatory effect. BEZ235 treatment, on the other hand, showed an effect on MYC expression only after 4 h, suggesting a more indirect effect on MYC gene expression. Double treatment of ATRA and BEZ235 resulted in significantly enhanced MYC mRNA downregulation, thus demonstrating important synergistic effects of the two treatments at the transcriptional level on the inhibition of MYC.

PAM pathway activity can increase MYC levels via two ways. First, mTORC1 promotes translation. This is achieved via phosphorylation of 4E-BP1, inhibiting its ability to negatively regulate the translation initiation factor 4E (eIF4E) and via phosphorylation of p70 S6 kinase 1 (S6K1), promoting transcription of ribosomal RNA and activation of eIF4B and eIF4F.[30,43] Second, PI3K and mTORC2 prevent degradation of MYC via phosphorylation and activation of AKT and inactivation of GSK3B, a mechanism that has been previously identified as a potential target in myelodysplastic syndrome and AML.[44,45]

Consistently we demonstrate that inhibition of different members of the PAM pathway as PI3K (ZSTK474), mTORC1/2 (WYE-125132) and PI3K/mTORC1/2 (BEZ235) decreased MYC levels and that inhibition of both mTOR complexes and PI3K by BEZ235 was most effective. Interestingly, while the addition of ATRA to PAMi clearly led to a further decrease of MYC protein, no impact of ATRA on activation marks of AKT or GSK3B was found, suggesting that MYC destabilisation via GSK3B is ATRA independent. On the other hand, inhibition of GSK3B attenuated the effects of BEZ235 on MYC phosphorylation and stability as well as subsequent differentiation and cell cycle/growth arrest, pointing towards a major effect of AKT permitted activation of GSK3B by PAM inhibition in AML, confirming and extending data of Imran et al.[40]

Of note, PAMi treatment led to a significant reduction of the mTORC1-regulated protein synthesis pathway via 4E-BP1 and RPS6 and this was further increased by ATRA. This finding can be explained by data from Gery et al.[39] who showed that the DNA damage inducible transcript 4 (DDIT4, RTP801) is an early retinoic acid response gene that is upregulated after retinoic acid treatment in KG-1a cells leading to mTOR inhibition as measured by a reduction of 4E-BP1 phosphorylation. DDIT4 was also significantly upregulated by ATRA and by ATRA and BEZ235 co-treatments 3 h after treatment in our data set. Giving the fact, that MYC translation is under control of EIF4B,[43,46] it seems plausible that MYC reduction by ATRA might be due to mTORC inhibition via DDIT4 or other pathways, next to transcriptional blockade. Additionally, it is worth mentioning that single mTORC1 inhibitors such as rapamycin have been shown to fail to produce a sufficient 4E-BP1 inhibition,[27,43] whereas this was demonstrated for PAMi in this study and by others.[27]

Over the past decade, it has become increasingly clear that different mutations and subpopulations prevent a long-term response to monotherapies. Therefore, various strategies have been developed to simultaneously target different genetic and epigenetic drivers or cell populations of AML and so to overcome resistance and prevent relapse.[47,48] The use of dual mTORC/PI3K inhibitors and their combination with ATRA promises several benefits. Dual mTORC/PI3K inhibition reduces the occurrence of negative PAM-feedback loops and unwanted AKT reactivation by targeting multiple core enzymes of the PAM pathway, while ATRA enhances S6K1 inhibition, one of the main activators of the insulin receptor substrate (IRS)1-AKT-feedback loop. Both, dual mTORC/PI3K inhibitors and ATRA, reduce MYC activities via partially different pathways, thereby dramatically lowering MYC levels in a synergistic manner. Despite the large genetic heterogeneity of AML, this mechanism seems to be universal and may also be relevant for the treatment of other cancers, as MYC still represents a challenging target.[49]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualisation: Sven Stengel, Kevin R. Petrie, Arthur Zelent and Tino Schenk. Methodology: Sven Stengel, Yordan Sbirkov, Tino Schenk, Clara Stanko, Veronica Gil and Tino Schenk. Writing – original draft preparation: Sven Stengel and Tino Schenk. Writing – review and editing: Sven Stengel, Kevin R. Petrie, Lukasz Szymański, Rafał Skopek, Paweł Kamiński, Annamaria Brioli, Arthur Zelent and Tino Schenk. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

High-throughput data sets have been deposited in the GEO with the accession code GSE160964 (https://www.ncbi.nlm.nih.gov/geo/).

ORCID

Rafał Skopek https://orcid.org/0000-0002-8669-3359
Łukasz Szymański https://orcid.org/0000-0003-0148-541X
Annamaria Brioli https://orcid.org/0000-0002-4072-7592
Tino Schenk https://orcid.org/0000-0003-3430-5876

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