Targeting AML Resistance with LY3009120-Sapanisertib and Ruxolitinib-Ulixertinib Combinations Demonstrate Superior Efficacy in *FLT3*, *TP53,* and *MUC4* mutations

Elham Gholizadeh (1); Ehsan Zangene (1); Alun Parsons (2); Mika Kontro (2),Caroline A. Heckman (2) and Mohieddin Jafari (1)

(1) Department of Biochemistry and Developmental Biology, Faculty of Medicine, University of Helsinki, Helsinki, Finland.
(2) Institute for Molecular Medicine Finland (FIMM), Helsinki Institute of Life Science, iCAN Digital Cancer Medicine Flagship, University of Helsinki, Helsinki, Finland.

* Corresponding author:

Mohieddin Jafari,

mohieddin.jafari@helsinki.fi

Abstract

Acute myeloid leukemia (AML) is a genetically heterogeneous malignancy characterized by the clonal expansion of myeloid precursor cells. Advances in genomic profiling have enhanced our understanding of AML pathogenesis, leading to the identification of recurrent mutations, including *TP53, FLT3, MUC4, RAS,* and *IDH1/2*. These mutations significantly influence treatment response and prognosis, with *TP53* mutations conferring poor outcomes and resistance to conventional therapies. Despite the advent of venetoclax-based regimens, resistance mechanisms persist, necessitating the development of novel therapeutic strategies. This study aims to investigate the efficacy of drug combinations for the treatment of AML using both *in vitro* AML cell lines and an *in vivo* zebrafish embryo xenograft model. Specifically, we focus on two drug combinations; the pan-RAF inhibitor LY3009120 combined with the mTOR inhibitor sapanisertib (designated as LS), and the JAK1/2 inhibitor ruxolitinib combined with the ERK inhibitor ulixertinib (designated as RU). The study integrates real-time cell viability assays, xenograft imaging, and genomic analyses to assess drug efficacy and explore correlations between treatment responses and mutational profiles, particularly *TP53, FLT3, and MUC4* mutations. Both combinations, LS and RU, demonstrated superior efficacy compared to venetoclax-based treatments in reducing cell viability across AML cell lines. The LS combination showed significant reductions in cell viability in MOLM16 and SKM cells, while RU exhibited comparable efficacy with lower toxicity profiles. In zebrafish embryos, the LS combination effectively inhibited the proliferation of xenografted human AML cells, as evidenced by decreased fluorescence signals, indicating cell death. The RU combination also disrupted survival signaling pathways, showing promise as a therapeutic strategy. Furthermore, a correlation was identified between drug response and mutational profiles, with *TP53, FLT3 and MUC4* mutations significantly influencing sensitivity to the LS and RU combinations. These findings support the further development of LS and RU as effective alternatives to current clinical regimens, with implications for personalized AML treatment.

Introduction

AML is a heterogeneous hematological malignancy characterized by the clonal proliferation of myeloid precursor cells, leading to impaired hematopoiesis[1]. The complexity of AML is underscored by its diverse genetic landscape, which includes a multitude of somatic mutations that drive the disease's pathogenesis and influence clinical outcomes[2]. Recent advancements in genomic technologies, particularly next-generation sequencing (NGS), have significantly enhanced our understanding of the genetic underpinnings of AML, facilitating the identification of recurrently mutated genes and their prognostic implications [3]. This genomic insight has paved the way for risk stratification and personalized treatment approaches, enabling clinicians to tailor therapies based on the unique molecular profiles of individual patients[4].

Among the various genetic alterations observed in AML, mutations in the *TP53* and *FLT3* genes have emerged as a critical factor in prognostication and treatment selection [5,6]. The *TP53* gene, located on chromosome 17p13.1, encodes the p53 protein, a pivotal tumor suppressor that plays a fundamental role in cellular responses to stress, including DNA damage and oncogenic signaling [7,8]. Mutations in *TP53* are prevalent across a wide spectrum of human cancers, with nearly half of all tumors exhibiting alterations in this gene [9,10]. In the context of AML, *TP53* mutations are associated with inferior responses to standard therapies and are linked to poor patient outcomes. The initial TCGA report showed 8% prevalence in newly diagnosed AML. The presence of mutant p53 not only compromises the efficacy of conventional chemotherapeutic agents but also suggests a need for novel therapeutic strategies that circumvent the reliance on this critical apoptotic pathway [11]. Additionally, *TP53* is the sole mutated gene identified in up to 75% of patients, while patients with co-occurring mutations have a decreased incidence of mutations in a number of AML-associated genes including *FLT3, NPM1, IDH1, IDH2, DNMT3A, WT1, RUNX1* and *RAS* [11–14]. Induction chemotherapy remains standard for fit AML patient but is less effective in *TP53*-mutated patients, with low response rates (20–40%), high relapse rates, and a median survival of 4–9 months [15,16]. However, untreated patients have even worse outcomes, highlighting the aggressive nature of *TP53*-mutated AML and the necessity of treatment [6,17]. *MUC4*, a mucin protein known for its role in various cancers, and examined its impact on treatment outcomes in AML [18]. *MUC4* is typically associated with promoting tumor growth and chemoresistance. Recent findings indicate that *MUC4* expression is significantly higher in AML patients than in healthy controls, correlating with poorer survival and shorter disease-free intervals [19]. *FLT3* is a gene frequently mutated in AML, making it a critical target for therapeutic intervention. Mutations in *FLT3*, particularly internal tandem duplications (ITD), are associated with poor prognosis and decreased overall survival in AML patients. The overexpression of *FLT3* leads to increased proliferation of leukemic cells, contributing to the disease's aggressive nature. *FLT3* inhibitors have been developed to target these mutations, but resistance often develops, necessitating biomarkers to stratify patients and guide treatment decisions [20].

Understanding the mutational background of AML, whether in patient samples or cell lines, is crucial for developing more effective and personalized therapies. Genetic alterations, such as mutations in TP53, FLT3, and MUC4, significantly influence treatment response and resistance. To address these challenges, cancer dependency and vulnerability analyses have emerged as powerful tools for identifying genetic interactions that are critical for cancer cell survival and proliferation. These approaches explore diverse pathways to pinpoint context-specific vulnerabilities. For instance, tools like SL-scan are designed to identify synthetic lethal interactions in both pan-cancer and cancer-specific contexts by integrating multiple layers of information, including mutation data and gene expression, to provide insights into personalized cancer vulnerabilities[21]. Additionally, large-scale perturbation screens, such as the DRIVE project, employ shRNA and CRISPR technologies to systematically map gene dependencies across various cancer types[21–23]. Computational tools, including SL-scan and APSiC, analyze these screens to identify synthetic lethal pairs and cancer dependencies, highlighting potential targets for personalized therapies. These techniques bridge the gap between large-scale genomic data and the identification of actionable therapeutic targets, facilitating the discovery of novel drug combinations and biomarkers for improved cancer treatment[24–26]. The challenge of drug resistance in AML, underscores the importance of considering the mutational background when designing therapeutic strategies.

Another challenge frequently facing in combinatorial threpay is the discrepancy of *in vitro* and *in vivo* outcomes. Zebrafish has emerged as a valuable in vivo model organism for cancer research, particularly in the context of xenograft studies. The transparent nature of zebrafish embryos allows for real-time imaging of cancer cell behavior, providing insights into tumor growth, invasion, and response to therapy [26–28]. Furthermore, the compatibility of zebrafish with high-throughput drug screening enhances its utility in evaluating the efficacy of therapeutic combinations. By transplanting human AML cell lines into zebrafish embryos, researchers can investigate the *in vivo* effects of drug treatments, thereby bridging the gap between in vitro studies and clinical applications.

Here, we investigated the efficacy of our two recent proposed drug combinations[26] in both AML cell lines and xenografted zebrafish embryos. Our research focuses on two specific drug combinations; the pan-RAF inhibitor LY3009120 in combination with the mTOR inhibitor sapanisertib (designated as LS), and the JAK1/2 inhibitor ruxolitinib in combination with the ERK inhibitor ulixertinib (designated as RU). We aimed to assess the efficacy of these combinations against a panel of AML cell lines, as well as in zebrafish embryos xenografted with human AML cell lines. The biological rationale for selecting these specific drug combinations is grounded in their mechanisms of action and the molecular pathways they target. The RAF-MEK-ERK signaling pathway is known to be hyperactivated in many cancers, including AML, and plays a crucial role in cell proliferation and survival [29]. By targeting this pathway with a pan-RAF inhibitor, we aim to disrupt the signaling cascade that promotes leukemic cell growth. Additionally, the mTOR pathway is integral to cell metabolism and growth; inhibiting this pathway in combination with RAF inhibition may yield synergistic effects, particularly in venetoclax-resistant AML subtypes. Furthermore, the

JAK/STAT signaling pathway has been implicated in the pathogenesis of AML, with ruxolitinib demonstrating efficacy in reducing leukemic cell proliferation [30]. The addition of ulixertinib, an ERK inhibitor, is expected to enhance the therapeutic effect by simultaneously targeting multiple signaling pathways involved in AML progression.

In our study, we employ real-time cell viability assays to assess the effects of the LS and RU combinations on AML cell lines, measuring cell viability over a 72-hour period. Additionally, we investigate the association between drug responses and mutational profiles. Moreover, we evaluate the *in vivo* efficacy of these combinations using zebrafish embryos xenografted with AML cells. This approach allows us to visualize the effects of the drug treatments on both the morphology of the embryos and the viability of the injected human cells. We anticipate that the results from these experiments provide valuable insights into the therapeutic potential of the LS and RU combinations, as well as their mechanisms of action. Although our experimental design was initially focused on drug response assay on monotherapy and nominal data mining[24], we observed that the cell lines studied carried key mutations, including *TP53*, *MUC4*, *FLT3*, and *HLA-B*. This approach highlighted the potential of ruxolitinib/ulixertinib (RU) and LY3009120/sapanisertib (LS) as effective combinations with favorable toxicity profiles.

Results

Efficiency of combination compared to single drugs

Our study employed several steps to understand drug actions and effects, thereby providing a multidimensional perspective on the development and optimization of the proposed anticancer therapies **(Fig. 1)**. We initiated our investigation by applying a RealTime-Glo (RTG) cell viability assay on ten AML cell lines over a 72-hour period, with measurements taken every 12 hours. This initial analysis aimed to evaluate the efficacy of two our recently proposed drug combinations—LY3009120 (a pan-RAF inhibitor) combined with Sapanisertib (an mTOR inhibitor), referred to as LS, and Ruxolitinib (a JAK1/2 inhibitor) combined with Ulixertinib (an ERK inhibitor), referred to as RU[26]. Then we also compared these two combinations with first-line treatments in AML. As expected, all the combination cases are more effective than single treatments. The results demonstrate that both combinations exhibit superior or comparable efficacy relative to standard venetoclax-based regimens (VC, VA, and VR)**(Fig. 2A)**, while also showing potential advantages in toxicity profiles based on xenograft model.

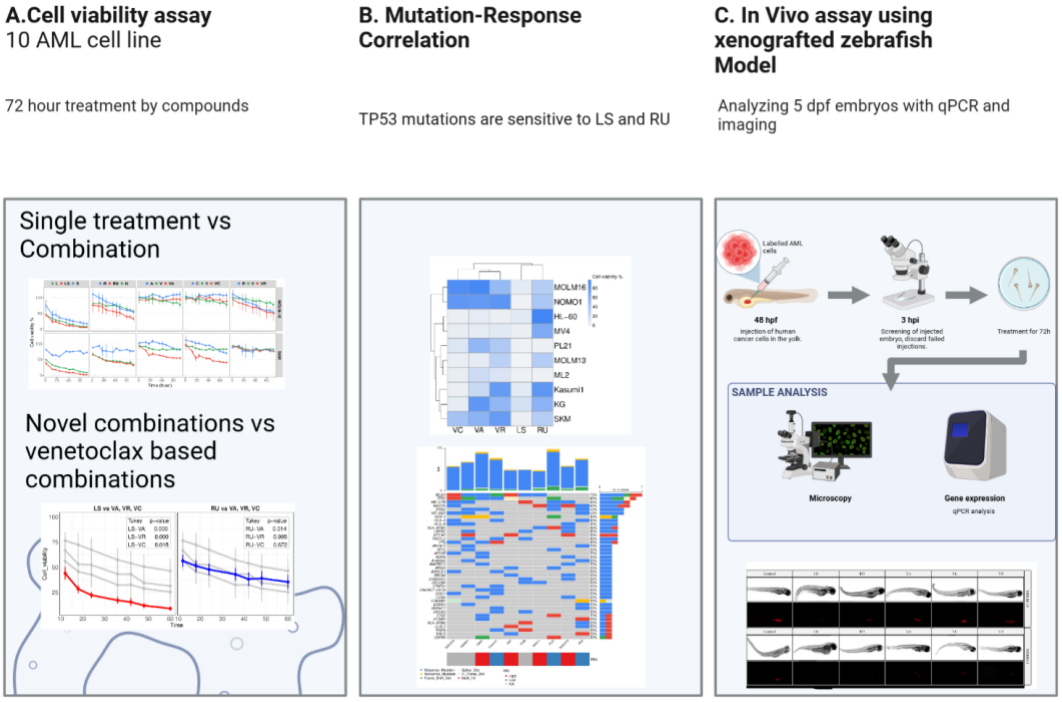

Figure 1. An overview illustrating the comprehensive methodologies employed in evaluating anticancer treatments through various experimental platforms: **A. Cell Viability Assay**: Testing the efficacy of treatments on 10 AML cell lines over 72 hours, comparing single-agent treatments with combination regimens. **B. Mutation-Response Correlation**: Assessing drug sensitivity associated with *TP53* and *FLT3* mutations in different cell lines, utilizing a heatmap to show the variation in response. **C. In Vivo Assay using Xenografted Zebrafish Model**: Employing a zebrafish model to investigate drug effects on labeled human cancer cells, analyzing gene expression and imaging post-treatment.

The efficacy of the LS combination is particularly pronounced, with a steep decline in cell viability observed consistently over the 72-hour observation period. This decline suggests a robust anti-leukemic effect that may be attributed to the simultaneous targeting of the RAF and mTOR signaling pathways, which are known to play critical roles in AML cell proliferation and survival. Similarly, the ruxolitinib/ulixertinib combination, targeting the JAK/STAT and ERK pathways respectively, show comparable efficacy to venetoclax-based combinations. Although, the toxicity of this combination was markedly lower[26], underscoring its potential therapeutic advantage over current clinical standards.

A detailed analysis of the cell-line-specific responses revealed that subsets of AML cells were particularly sensitive to the LS combination, achieving significantly greater reductions in cell viability **(Fig. 2B)**. Notably, MOLM-16 and SKM cells demonstrated heightened sensitivity, with sustained suppression of cell viability across the 72-hour observation window and the viability reduced to below 25%. This suggests that targeting both RAF and mTOR pathways in AML provides synergistic effects, particularly in venetoclax-resistant subtypes. The response to the RU combination, although more uniform across all tested cell lines, also indicated potential for personalized treatment strategies. When compared to single-drug therapies, both combinations yielded superior results in reducing AML cell viability, further validating their potential efficacy in clinical settings. The results suggest that targeting specific pathways using these novel drug pairs could be more effective in overcoming resistance mechanisms commonly associated with AML treatments. As shown in **Fig. 2A** , statistical comparisons using Tukey's test indicated no significant differences between RU and VR/VC combinations ($p > 0.6$), whereas RU significantly outperformed VA ($p = 0.014$).

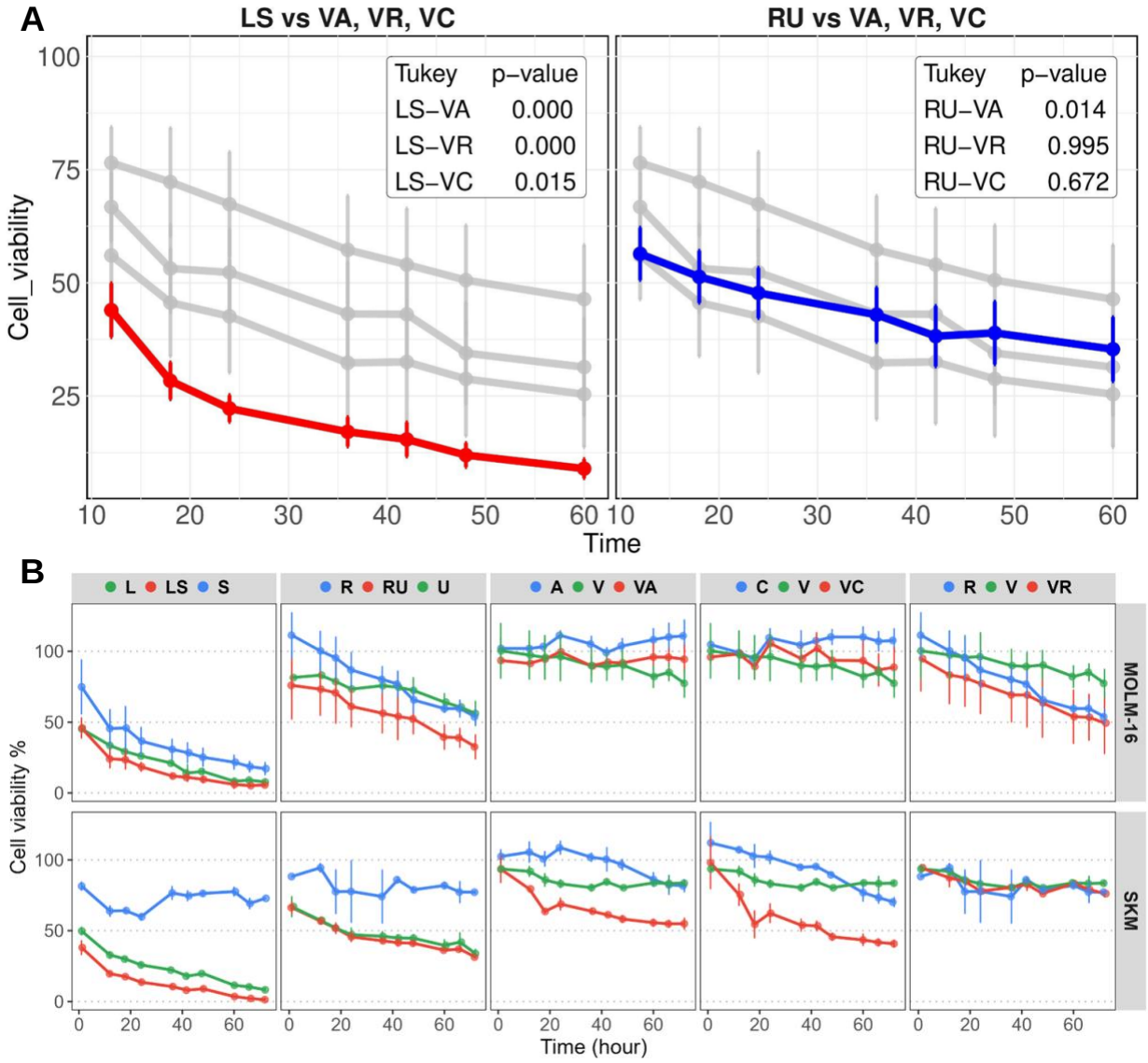

Figure 2. Efficacy and statistical analysis of LS and RU combinations compared to venetoclax-based regimens in AML cell lines. (A) Statistical comparison of LS and RU combinations against venetoclax-based regimens in ten AML cell lines: Tukey's post-hoc test was performed to compare LS and RU to venetoclax-based regimens. **(B)** Comparison of LS and RU combinations with single agents and venetoclax-based regimens in SKM and MOLM-16 AML cell lines. Cell viability (%) was monitored over 72 hours in SKM and MOLM16 cells. Panels compare single agents against their combination. Error bars indicate standard deviations.

Drug Sensitivities Associated with Mutations

To further investigate the relationship between drug responses and mutational profiles in AML cell lines, we analyzed drug efficacy data for LS and RU combinations in conjunction with the genomic mutation landscape of the ten tested AML cell lines. We identified some key mutations such as *TP53, FLT3*, and *MUC4*, which are known to influence treatment responses in AML[31]. The LS combination demonstrates the highest efficacy, with

significant reductions in cell viability observed in MOLM-16 (94.40%) and NOMO-1 (80.94%). Similarly, RU exhibited robust effects in MOLM-16 (97.67%) and NOMO-1 (63.36%), confirming the effectiveness of these combinations in certain AML subsets **(Fig. 3A)**.

In contrast, other cell lines, such as HL60 and Kasumi1, show minimal response, highlighting heterogeneity in drug sensitivity across AML models. Hierarchical clustering of AML cell lines based on mutational data **(Fig. 3B)** reveals distinct genomic patterns. For instance, MOLM-16 and NOMO-1 are clustered separately, associated with poor responses to venetoclax-based regimens while decreasing viability in two proposed combinations. Notably, both cell lines harbor mutations in *TP53*, a critical gene associated with resistance to venetoclax-based treatments. *TP53* is recognized as the "guardian of the genome," playing a vital role in maintaining cellular integrity during genomic stress[32]. Interestingly in our recent work, we have identified *TP53* as a direct target for samples treated with both singles and combination of ruxolitinib/ulixertinib in lysate cells using the CoPISA method[33]

Moreover, we identified MUC4 mutations in the context of our study, which may influence the response to leukemic cells, further complicating treatment efficacy. MUC4, a mucin protein, has been extensively studied for its role in various solid malignancies, where it is linked to tumor growth, proliferation, adhesion, invasion, inhibition of apoptosis, and chemoresistance [18]. However, its expression and clinical significance in AML have not been thoroughly explored until recently. MUC4 expression levels are significantly elevated in the bone marrow of AML patients compared to healthy controls. This overexpression is associated with worse survival outcomes and shorter disease-free survival, making MUC4 an independent prognostic marker for AML, also highlighting its importance in understanding disease progression and treatment response [19].

The other mutation in MOLM-16 and NOMO-1 is human leukocyte antigen *HLA-B* which influences the immune system's ability to recognize and respond to leukemic cells, thus playing a role in both the susceptibility and progression of AML [34]. Importantly in our previous study, this gene has been targeted by both combinations and single drugs in lysate and living cells. The presence of these mutations underscores the necessity of targeting specific pathways using these drug pairs, which could be more effective in overcoming resistance mechanisms commonly associated with AML treatments.

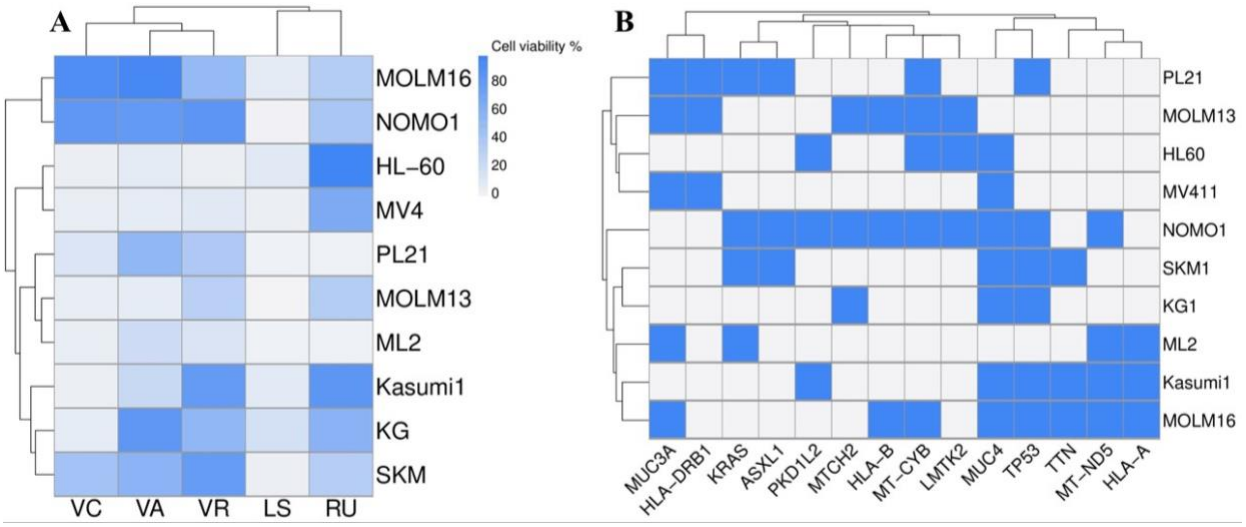

Figure 3. Drug response and mutational clustering in AML cell lines. (A) Cell viability after treatment with combinations: Ten AML cell lines were treated with venetoclax-based combinations and two novel combinations. Cell viability has been measured following 72 h. **(B)** Hierarchical clustering of mutational profiles: AML cell lines were grouped based on shared genomic mutations. The blue elements represent the mutations in the corresponding genes and cell lines.

Genomic Alterations and Proteogenomic Correlations with Drug Sensitivity

Our comprehensive analysis delineates distinct genomic landscapes in AML cell lines that correlate with cellular response to targeted therapies. Based on recent studies, AML cells can be categorized as Mito-high and Mito-low subtypes based on mitochondrial protein profiling, with significant implications for treatment efficacy [35]. Mito-high subtypes are characterized by a heightened expression of mitochondrial proteins, which correlates with aggressive disease phenotypes and poor clinical outcomes, particularly a lower remission rate and shorter overall survival post-intensive induction chemotherapy [35]. This pivotal insight suggests that mitochondrial biogenesis and function play crucial roles in cellular metabolism and survival, substantially influencing treatment responses.

In our study, the Mito-high AML cell lines **(Fig. 4)**, specifically SKM-1, KG-1, MV4-11, and MOLM-13, exhibit enhanced sensitivity to LS and RU drug combinations. These cell lines demonstrate a notable response to these treatments, highlighting their potential in managing patients with otherwise poor prognosis. Specifically, the KG-1 and SKM-1 cell lines show resistance to venetoclax-based treatments, as indicated by cell viability results.

This integration of proteomic data with genomic insights provides a nuanced understanding of AML pathophysiology, emphasizing the potential of mitochondrial profiling to predict and enhance therapeutic outcomes in AML. This comprehensive method not only augments our ability to address the heterogeneity of AML but also facilitates the development of personalized treatment protocols that improve patient outcomes in this challenging malignancy.

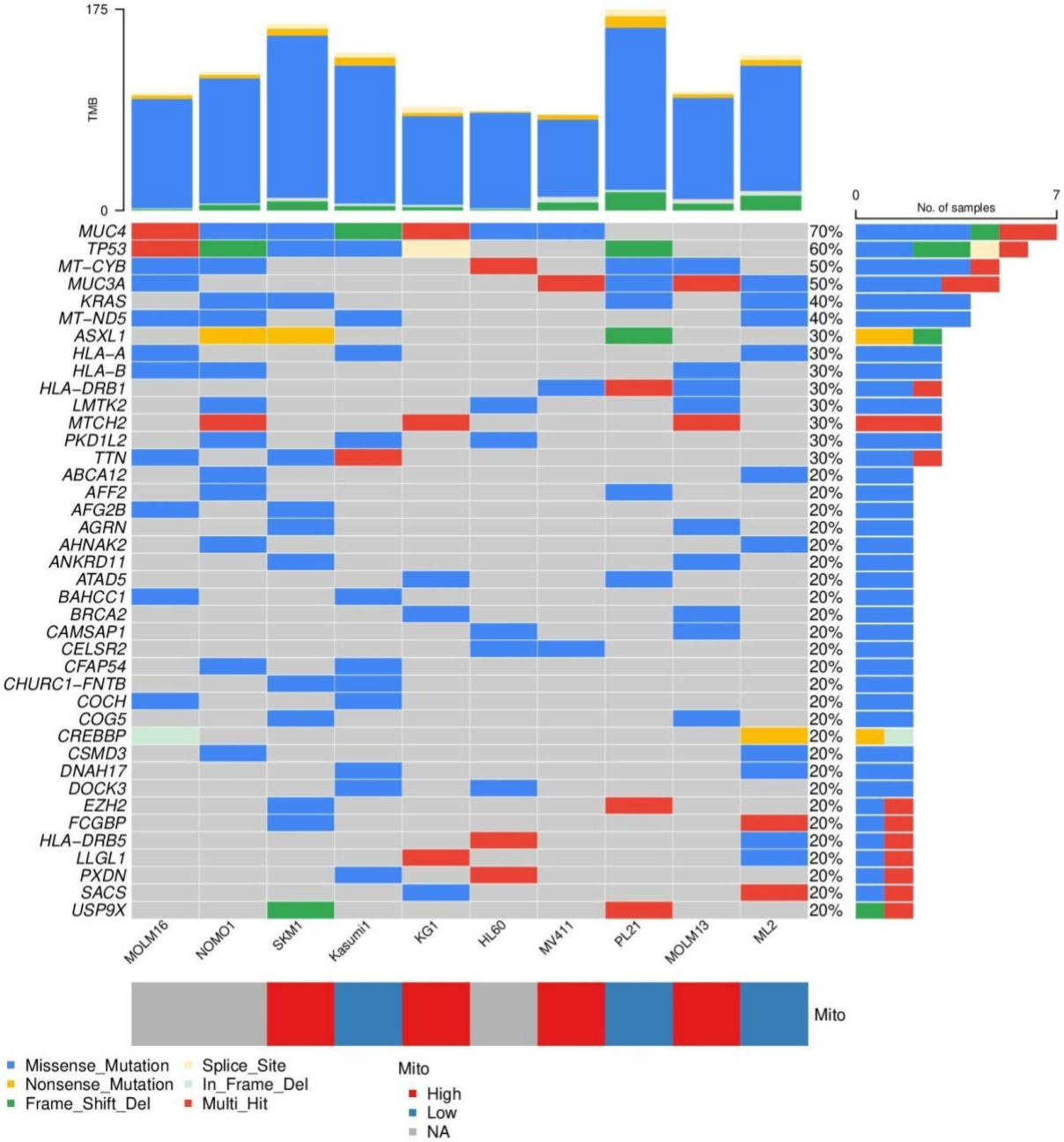

Figure 4. OncoPrint plot showing the most recurrent somatic mutations in 10 AML cell lines. Rows correspond to genomic variants, columns correspond to samples, variants are ranked by mutations frequency (left). Alterations are color-coded (bottom). Frequency and type of alterations for each individual sample are shown at the top. Cell lines are classified as Mito low and high based on resources.

APSiC Cancer Gene Dependency Analysis

To further investigate the significance of genomic alterations in the selected AML cell lines, we utilized APSiC, a cancer gene dependency analysis tool. APSiC integrates multi-omics and cancer vulnerability datasets, primarily sourced from DepMap, and provides a user-friendly online interface[22]. Among the hypermutated genes identified in the chosen AML cell lines, TP53, MUC4, and HLA-B were notably overrepresented. Using APSiC, we evaluated these genes across both pan-cancer and leukemia-specific contexts.

As illustrated in **Fig. 5**, TP53 emerged as a significantly mutated cancer gene in the pan-cancer analysis, with a highly significant p-value of 7.3e-56. However, in the leukemia-specific context, APSiC did not reveal significant missense mutation enrichment ($p = 0.37$). Conversely, HLA-B, a gene with hypermutation potential, exhibited a p-value of 0.054 in the pan-cancer analysis, but the leukemia dataset lacked sufficient samples for statistical validation.

The discrepancies observed in APSiC analyses between pan-cancer and AML-specific contexts underscore the necessity of context-dependent experimental studies to identify optimal targeted therapies, whether as single agents or in combination. These findings emphasize the critical importance of tailoring therapeutic strategies to the specific genetic backgrounds of distinct cancer types, complemented by drug response analyses pariticularly in vivo models, to elucidate resistance mechanisms and improve treatment outcomes.

Image-based Drug Efficacy in Xenografted Zebrafish Embryos

To extend our investigation, we evaluated the efficacy of the LS and RU combinations in a zebrafish model, utilizing MOLM-13 and NOMO-1 cell lines, harboring *FLT3* and *TP53* mutations. We specifically chose these cell lines due to the critical role these mutations play in AML. In previous work, we applied LS and RU combinations to various patient samples, predominantly characterized by FLT-3 mutations[26]. Building on this foundation, we continued to explore the effectiveness of these combinations, as well as venetoclax-based regimens, using the same cell lines in zebrafish models to deepen our understanding of their therapeutic potential.

The experimental design involved the injection of zebrafish embryos with MOLM-13 or NOMO-1 cells, which were pre-labeled with CellTrace Far Red dye. Following a 72-hour incubation period, the embryos were subjected to treatment with a range of drug combinations and single drugs. The efficacy of these treatments was assessed through both brightfield and fluorescence microscopy, allowing for the visualization of drug effects on the morphology of the zebrafish embryos as well as the viability of the injected human cells. As shown in **Fig. 6**, the brightfield microscopy images provided a comprehensive overview of the overall morphology of the zebrafish embryos after treatment with the various drug combinations. The embryos exhibited distinct morphological changes, which were indicative of the effects of the treatments on both the host organism and the xenografted human cells.

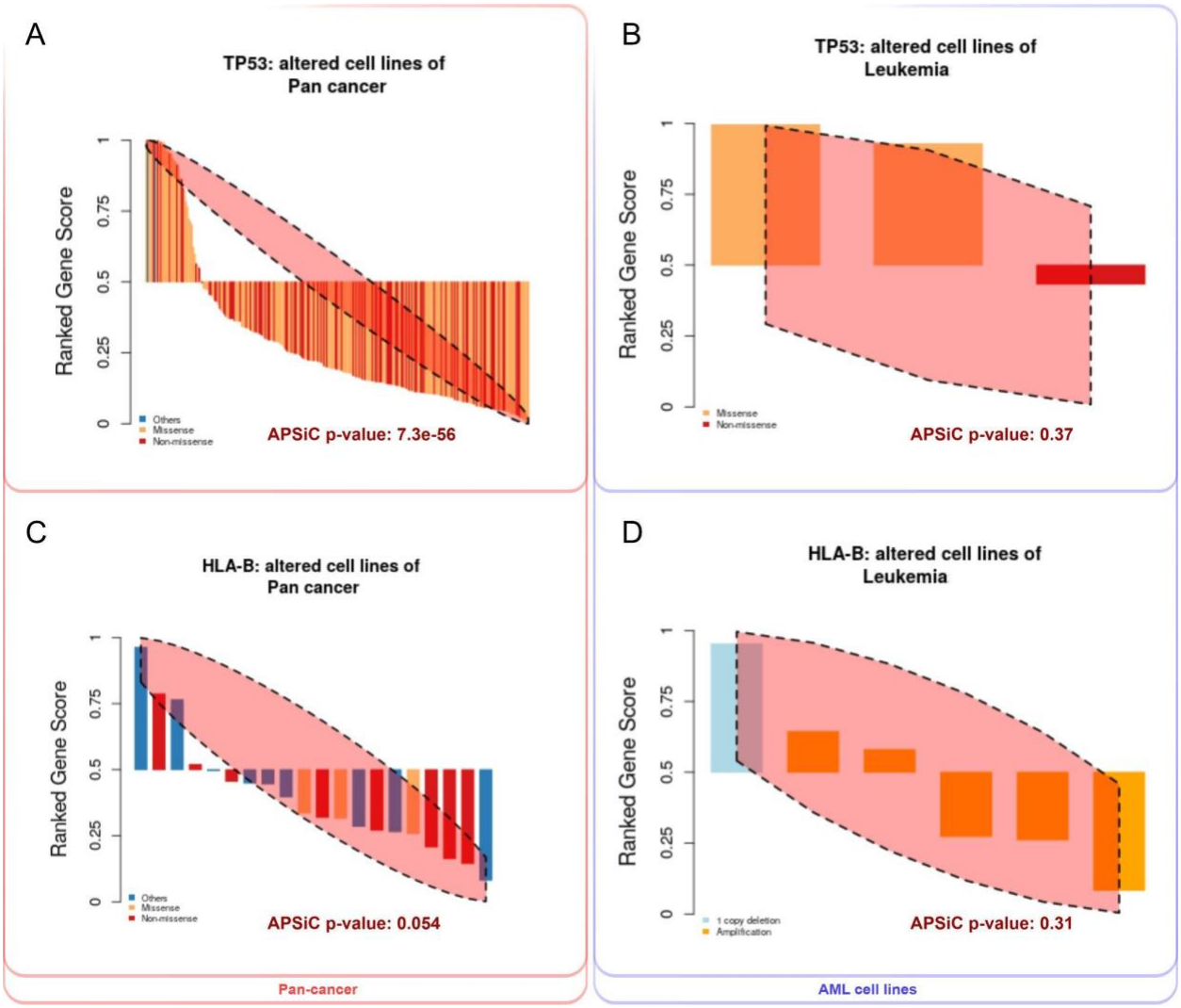

Figure 5. Waterfall plot of CRISPR gene dependency scores (ATARIS scores) for selected cancer cell lines. The y-axis represents cell viability based on CRISPR gene dependency scores, while the x-axis shows ranked cell lines of the cancer types relevant to the study. The dependency scores illustrate the sensitivity of AML cell lines to gene knockouts, highlighting key vulnerabilities in specific genomic contexts. **(A)** TP53 altered cell lines of the pan cancer context. **(B)** TP53 altered cell lines of the Leukemia context. **(C)** HLA-B altered cell lines of the pan cancer context. **(D)** HLA-B altered cell lines of the Leukemia context.

Embryos treated with the combination of LS demonstrated a notable reduction in the fluorescence signal from the MOLM-13 and NOMO-1 cells compared to the untreated controls. The fluorescence microscopy images revealed a significant decrease in the number of viable leukemia cells, suggesting that the LS combination effectively inhibited cell proliferation and induced cell death. The treatment with RU led to a notable change in the fluorescence intensity of human leukemia cells. This indicates that the RU combination effectively disrupts the signaling pathways linked to cell survival and growth. The combinations of venetoclax exhibited a different profile of efficacy. Zebrafish embryos treated with VA showed a moderate decrease in fluorescence intensity, with approximately a 50% reduction in the number of viable MOLM-13 and NOMO-1 cells. The VC combination

demonstrated a synergistic effect on the reduction of leukemia cell viability. Fluorescence analysis revealed a remarkable decrease in fluorescence intensity, with an estimated reduction of 75% compared to untreated controls. This suggests that the VC combination effectively enhances the anti-leukemic activity of venetoclax, potentially through the induction of apoptosis and inhibition of cell proliferation. Finally, the combination of VR exhibited the most pronounced effects among the drug combinations tested. Fluorescence microscopy revealed a dramatic decrease in the number of viable human leukemia cells.

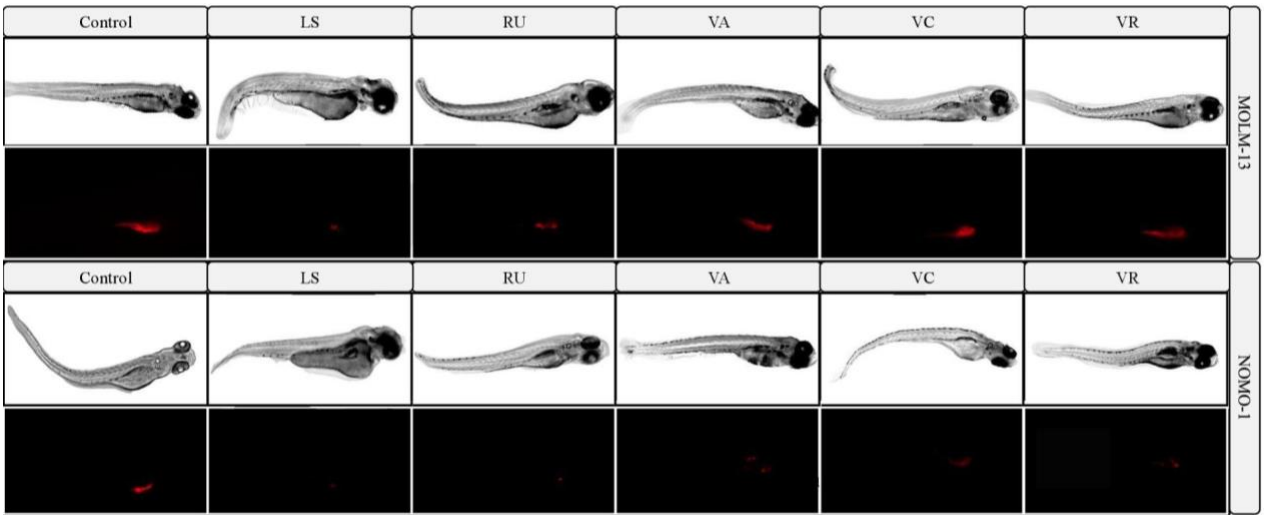

Figure 6. Visualization of drug efficacy in MOLM-13 and NOMO-1 xenografted zebrafish embryos. Brightfield and fluorescence microscopy images of zebrafish embryos injected with MOLM-13 or NOMO-1 cells labeled with CellTrace Far Red, followed by 72-hour incubation and treatment with various drug combinations. Treatments include LY3009120 + sapanisertib (LS), ruxolitinib + ulixertinib (RU), venetoclax + azacitidine (VA), venetoclax + cytarabine (VC), and venetoclax + ruxolitinib (VR). The upper rows display the overall morphology of the zebrafish, while the lower rows highlight fluorescence signals from labeled human cells, reflecting drug effects.

Gene expression-based Drug Efficacy in Xenografted Zebrafish Embryos

The impact of single and combinations of proposed and first-line combinations on the expression of genes, including *RPL0, GAPDH,* and *HNRNP* is investigated in the MOLM-13 and NOMO-1 cell lines which have the most important mutations in AML, *FLT3* and *TP53* **(Fig. 7)**. In the MOLM-13 xenografted embryos, differential gene expression patterns are observed across the treatments. The LS and RU combinations result in a substantial reduction in the expression levels of all three genes, indicating potent suppression of cellular activity in the treated cells. Specifically, LS treatment leads to a marked decrease in *HNRNP* and *RPL0* expression compared to the single-agent treatments, with *GAPDH* showing similar trends.

RU treatment further suppresses the expression of all three genes, achieving the lowest overall expression levels among the tested combinations. Comparatively, venetoclax-based combinations (VA, VC, and VR) display moderate reductions in gene expression, with VR demonstrating the strongest effect among the venetoclax regimens. In the NOMO-1

xenografted embryos, a similar trend is observed, with LS and RU combinations showing the greatest suppression of gene expression. *HNRNP* and *RPL0* are significantly downregulated following LS treatment, mirroring the MOLM-13 results. RU treatment results in consistent reductions across all genes, reinforcing its efficacy in targeting NOMO-1 cells. Venetoclax-based combinations show variable effects, with VR producing greater suppression compared to VA and VC, though none match the efficacy of LS or RU.

Across both xenograft models, the LS and RU combinations consistently achieve the most significant suppression of gene expression. The venetoclax-based regimens, while effective, are less potent in reducing gene expression, particularly in MOLM-13 cells, which harbor *FLT3* mutation. These results suggest that LS and RU combinations exert superior anti-leukemic effects, potentially through enhanced downregulation of key metabolic and structural genes *in vivo*. The implications of these findings underscore the potential of these novel combinations in targeting specific mutations such as *FLT3, TP53*, and *MUC4* associated with treatment resistance in AML, paving the way for improved therapeutic strategies in clinical settings.

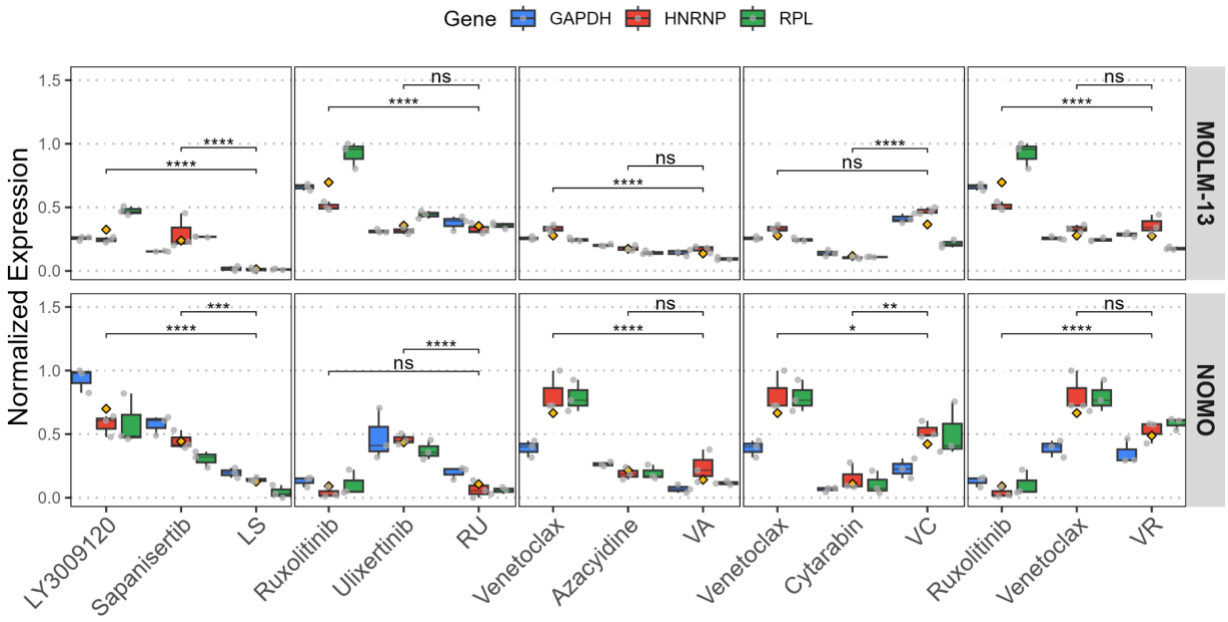

Figure 7. Gene expression of human cells in xenografted zebrafish. This figure illustrates the normalized expression levels of three genes—GAPDH, HNRNP, and RPL— across various drug treatments in MOLM-13 and NOMO-1 cell lines. Human cells were injected into 3 days post-fertilization (dpf) zebrafish embryos, followed by a 72-hour treatment with various drugs and drug combinations. Each panel represents a different treatment, showing the gene expression variability within treatment groups. Statistical significance between groups is denoted by asterisks directly above comparisons, where **'ns'** indicates no significant difference and increasing numbers of asterisks reflect higher levels of significance (*p < 0.05, ***p < 0.001, ****p < 0.0001).

Discussion

The findings of this study provide compelling evidence for the efficacy of our proposed drug combinations, LS and RU, in targeting AML, particularly in the context of resistance mechanisms associated with *TP53, FLT3* and *MUC4* mutations. Our research demonstrates that both combinations not only surpass the efficacy of venetoclax-based regimens but also exhibit favorable toxicity profiles, positioning them as promising candidates for further clinical evaluation.

The superior efficacy of the LS combination against AML cell lines, particularly MOLM-16 and SKM, underscores the potential of targeting both the RAS-RAF-MEK-ERK (MAPK) and mTOR pathways in overcoming monotherapies in oncology [36,37]. The robust decline in cell viability observed with the LS combination suggests a synergistic effect, likely due to the simultaneous disruption of multiple signaling cascades that are critical for leukemic cell proliferation and survival. This is particularly relevant given the complexity of AML's genetic landscape, where mutations in key oncogenes and tumor suppressors, such as *TP53, FLT3 and MUC4*, pose significant challenges for previous therapies. The results align with previous studies indicating that combined inhibition of these pathways can effectively mitigate the survival advantages conferred by such mutations, thereby enhancing treatment responsiveness[38]. Similarly, the RU combination, targeting the JAK/STAT and ERK pathways, presents a comparable efficacy to current venetoclax regimens. The ability of RU to disrupt survival signaling pathways while maintaining a favorable safety profile is particularly noteworthy, as it addresses a critical concern in AML treatment where patients often experience significant treatment-related morbidity. The findings advocate for the exploration of these combinations in clinical trials, particularly in populations with known *TP53* and *FLT3* mutations, where traditional therapies have historically yielded poor outcomes.

The recent study by Jayavelu et al. highlights the need for a paradigm shift in how we categorize AML subtypes[35]. The authors emphasize that mutation-centric precision medicine currently faces significant limitations and propose a classification framework based on proteomic expression profiles. Such an approach offers a novel perspective for predicting treatment responses. Their findings reveal that Mito-high cell lines are associated with poorer outcomes, including lower remission rates and shorter overall survival following intensive induction chemotherapy. Although our dataset is not extensive enough to provide additional evidence for the Mito-high/Mito-low hypothesis, ironically, our proposed drug combinations align with this context by not relying solely on genomic backgrounds. These combinations were developed through nominal data mining of monotherapy drug response assays at the phenotypic level[24–26]. Interestingly, we observed a correlation between mutational backgrounds and cell viability outcomes, which reinforces the validity of our approach.

Overall, incorporating diverse biological data across different phenotypical and molecular levels[39]—such as drug response, protein expression profiles, and genomic data—enables a more comprehensive identification of the unique vulnerabilities of individual tumors. This integrative approach holds significant potential for improving treatment outcomes, particularly for patients with complex mutational landscapes. By uncovering actionable targets that may not be apparent through genomic analysis alone, this strategy paves the way for the development of more effective and personalized treatment protocols.

Method

Cell culture

Human myeloid leukemia cell lines (HL60, Kasumi-1, KG-1, ML-2, MOLM-13, MOLM16, MV4-11, NOMO-1, PL-21, SKM) were obtained from (Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures GmbH). All cells were grown in RPMI-1640 (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% or 20% FBS (Gibco), 1% L-Glutamine (Gibco), and 1% Penicillin-Streptomycin (Gibco). To prepare the cell suspensions for injection, cells were washed three times with PBS at 300 g for 3 minutes to remove any residual culture medium. For imaging purposes, cells were stained using CellTrace Far Red (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. At the final step of dying cells, cells were then suspended in media at a concentration of 50×10^3 cells/µl. For microinjection, 4 nL of this suspension was injected, resulting in approximately 200 cells per embryo.

Zebrafish larvae microinjection and drug administration

The age of embryos is indicated as hours post-fertilization (hpf) and days post-fertilization (dpf). The larvae were grown at 28.5 °C in an embryonic medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 and 0.33 mM MgSO4; Sigma-Aldrich). Two dpf, wild-type zebrafish (Danio rerio) larvae (from AB strain) were used. Embryos were dechorionated, anesthetized with 0.04 % tricaine, and microinjected through the yolk sac with 4 nL of cells. ~1000 cells were injected per fish. Fish were transferred into a 24-well plate (five fish per well) containing 1.5 mL of fresh embryonic medium with desired drug concentrations, and incubated for three days at 34 °C. The zebrafish larvae were treated with single and combinations as bellow concentration: LY3009120 500nM, Sapanisertib 500nM, Ruxolitinib 3uM, Ulixertinib 3uM, venetoclax 50nM, azacitidine 300nM, cytarabine 1000nM. Drug concentrations were chosen based on our previous in vitro drug testing as well as on the cytotoxicity test for the zebrafish larvae. Drugs were diluted in the embryonic medium and DMSO was used as the negative control. Twenty fish were used for each drug, where nine fish were pooled together to provide a sufficient signal during PCR amplification and the rest were used for imaging.

Drug combination testing

The compounds were dissolved in dimethyl sulfoxide (DMSO) and dispensed on 384-well plates (Corning, Corning, NY, USA) for RTG and 24-well plate for zebrafish treatment. Drug concentrations were selected based on the previous study and cytotoxicity test for the zebrafish larvae. DMSO was used as a negative control by the concentration of 0.01% in RTG and 0.1% in the zebrafish experiment. The drug concentrations are as below: LY3009120 500nM, Sapanisertib 500nM, Ruxolitinib 3uM, Ulixertinib 3uM, venetoclax 50nM, azacitidine 300nM, cytarabine 1000nM

Fixation and imaging

Following 72h treatment, larvae were collected and fixed with 4 % paraformaldehyde and mounted on glass slides in SlowFade™ Gold antifade reagent (Invitrogen) for imaging. Zebrafish were imaged with a Zeiss Axio Imager (Carl Zeiss AG, Oberkochen, Germany), and the tumour area was analyzed using ImageJ. Zebrafish injections were conducted at the Zebrafish Unit at the University of Helsinki under ethical permission (ESAVI/13139/04.May 10, 2017). All experiments were repeated three times independently, with 15–23 larvae per group.

Statistical analysis

Values are given as means ± standard deviations. To determine the statistical significance, we performed non-paired Student's T-test, and one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. We set statistical significance to $P \leq 0.05$. The mutation lists for cell lines are downloaded from DepMap.

Cell viability analysis using RealTime-Glo™

AML cell lines were seeded on 384-well plates (Corning) containing desired concentration of drugs with three replicates. The number of cells per well was desired based on a linearity test as described in manual for continues-read experiment. In brief, cells were plated at increasing cell densities in the presence of 1X RealTime-Glo™ reagent, and cell viability was monitored by measuring luminescence every 12 hours for 72 hours. Based on the results the number of cells which remained linear in 72 h was selected (KG-1, MOLM16, HNT, Kasumi-1 with 5000 cells per well and HL60, ML-2, MOLM-13, MN4-11, NOMO-1, PL-21, SKM with 1500 cell per well). Following the linearity test, the desired number of cells per well were adjusted to final volume of 25 ul and were incubated with compounds for 72h at 37°C and 5% CO2. Cell viabilities was assessed using the CellTiter-Glo assay (Promega), and the luminescence signal was measured using a PHERAstar FS plate reader (BMG LABTECH, Ortenberg, Germany). Cell viability was measured at timepoints of 0, 12, 24, 36, 48, 60, and 72h.

Primer design and real-time quantitative PCR

Sequences of candidate genes were searched from GenBank for PCR primer designing using NCBI with Tm 59–62°C, primer length 18–22 bp, guanine-cytosine content 50–60% and amplicon length 80–220 bp. The secondary structures of primers were checked using Gene runner. We designed three primers for human genes and four for zebrafish. Quantitative PCR was performed using Lightcycler-FastStart DNA Master SYBR Green 1 and Lightcycler480 (Roche) according to the manufacturer's instructions. The qPCR was performed in 10 µl with a primer concentration of 1 µM, 10 ng cDNA and 1× SYBR Green qPCR Master Mix (Roche). The amplification programme consists of an initial denaturation

step at 95° C for 12 min and 40 cycles of denaturation at 95° C for 10s, annealing at 61°C for 15 s and extension at 72° C for 15 s. A total of two biological replicate samples were analysed in duplicates (technical duplicates). Since the PCR efficiency of the primer pair limitedly affects the analysis of gene expression stability across samples, the PCR efficiencies of the primers used were above 1·8, as determined by qPCR with serially diluted pooled cDNA samples (10^6, 10^5, 10^4, 10^3 dilutions), which was slightly beyond the recommended range 1,90 to 2,05.

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