

Axin-2/BCL2 axis regulates apoptosis upon targeting TAM kinase receptors in leukemia

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Abstract

Leukemia is the clonal expansion of abnormal undifferentiated white blood cells which outnumber the normal cells causing severe platelet and red blood cells (RBCs) depletion. The overexpression of TAM (Tyro3, Axl and MerTk) Kinase receptors has been associated with leukemogenesis. The expression of TAM Kinase receptors has been known in the representative leukemic cell lines selected for this study. This study was undertaken to investigate the link between downstream effectors of canonical WNT, PI3K/AKT, RAS/MAPK/ERK and intrinsic apoptotic pathway to propose a targeted therapy for leukemia. Proliferation assay carried out at concentrations $2\times$ (0.3, 0.5, 1, 1.5 μ M) of TAM Kinase receptor inhibitors revealed marked proliferation inhibition in acute myeloid leukemia (AML) in comparison to chronic myeloid and acute lymphocytic leukemia. The transcriptional analysis with two-step real time PCR and translational analysis via western blotting highlighted Axin-2/BCL2 axis to be active behind the mitochondrial apoptosis. Our findings show that upon targeting TAM Kinase receptors Axin2 and BCL2 expression changed contrariwise reflecting a potential canonical WNT involvement in mitochondrial apoptosis. A high Axin-2 and low BCL2 in Kasumi-1 and a low Axin-2 with a high BCL2 in K562 and Jurkat supports the aforementioned WNT involvement in intrinsic apoptotic signaling. A high BCL2 might have induced cell cycle arrest in Molm-13. A comparative analysis of the study strengthened the notion that resistance and induction of proliferation inhibition in leukemic cell lines depends upon the Axin-2/BCL2 axis upon targeted TAM kinase receptor inhibition therefore targeting this axis along with TAM Kinase receptors can help overcome therapy resistance and relapse.

INTRODUCTION

Leukemia is a hematologic malignancy characterized by cell type and progression into four primary categories including Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML), Acute Lymphocytic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL)[1]. Most of the different forms of leukemia involve chromosomal translocations and genetic mutations which serve as oncogenes leading to constitutive activation of several different pathways promoting enhanced proliferation and survival[2]. Flt3/ITD (Flt3 gene Internal Tandem Repeat) mutation in AML and BCR/ABL (Breakpoint Cluster Region-Abelson murine leukemia viral oncogene homolog) translocation in CML and ALL drive oncogenesis in most of the leukemic patients[3, 4]. Leukemogenesis is caused by aberrant cytokines, growth factors and protein tyrosine kinases which are involved in oncogenic signaling[5, 6]. Midostaurin and quizartinib for tyrosine kinases in AML, imatinib, nilotinib, dasatinib and ponatinib in CML etc. are initially very effective but later therapy resistance leads to relapse[7–9]. Isocitrate Dehydrogenase (IDH) inhibitors promote differentiation in various types of AML, inhibiting clonal expansion of undifferentiated cells to some extent[10, 11]. Many targeted therapies have been introduced to induce or restore apoptosis in leukemic cells[12, 13]. TAM (Tyro3, AXL, MerTk) Kinase receptors (TKRs) are frequently overexpressed in leukemia offering therapy resistance and relapse[14, 15]. Targeting these receptors has shown significant improvement in various types of leukemia[16, 17]. Intrinsic apoptotic pathway or programmed cell death is necessary for the removal of damaged cells[18]. This pathway is often dysregulated in leukemia offering resistance to therapy[19]. Oncogenic signaling pathways have been associated with

BCL2 (B Cell Lymphoma 2), the main component of this pathway[19, 20]. Most often the expression of BCL2 is found to be elevated in different types of leukemias[21]. This overexpression is linked to aberrant signaling pathways inhibiting apoptosis[21, 22]. Anti-apoptotic signaling is a hallmark of leukemia as a result of which damaged cells become senescent[23, 24]. These senescent cells mostly behave like stem cells contributing to relapse[25, 26]. Leukemic stem cells have the potential to initiate tumors due to immense replicative potential and immune evasion[27]. The main pathways involved in leukemogenesis include WNT (Wingless-related integration site), RAS/MAPK (Rat sarcoma / Mitogen-Activated Protein Kinase pathway) and PI3K/AKT (Phosphoinositide 3-kinase / Protein Kinase B pathway) pathways[28–30]. The aberrant expression of these signaling cascades has been linked to TAM kinase receptor over expression[15, 30]. Therefore, the rationale of the current study was to investigate how apoptosis induction differs in different types of leukemia upon targeting TAM kinase receptors and how it is linked with downstream targets of oncogenic signaling. Table 1 contains the summary of data reported so far on TAM Kinase receptors and its link with signaling pathways. WNT signaling has been reported to be involved in therapy resistance and relapse. Table 2 concludes our experimental results highlighting Axin-2/BCL2 axis behind cytotoxicity, transcriptional and western blotting profiles. Axin-2 (Axis inhibition protein 2) overexpression enhances apoptosis and its suppression by silencing reduces apoptosis[31–34]. Our study has revealed that targeting TAM kinase receptors regulates apoptosis via Axin2/BCL2 axis. A high axin-2 and low BCL2 expression in Kasumi-1 induces apoptosis. In Molm-13 (Myeloblast/Occasionally Lymphoblast-like Malignant cell lines) a high BCL2 along with high c-Myc (cellular Myelocytomatosis oncogene) and Axin-2 upon targeting AXL induces cell cycle arrest and prevents apoptosis. BCL2 and c-Myc function as protooncogenes[35]. BCL2 abrogates the apoptosis inducing activity of c-Myc while keeping its mitogenic functions[35, 36] which strengthens our results that despite high c-Myc expression, BCL2 prevents apoptosis in Molm-13 thereby inducing cell cycle arrest. In K562 and Jurkat cells a low axin-2 and high BCL2 expression prevents apoptosis via ERK1/2 (Extracellular Signal-Regulated Kinase 1 and 2) or PI3K/AKT pathway since the effectors of both of these pathways are severely downregulated upon targeting TAM kinase receptors.

MATERIALS/METHODS

Cell Lines

All the cell lines were kindly provided by CRM, Aga Khan University. Molm-13 and Kasumi-1 cells were maintained in RPMI 1640 (Roswell Park Memorial Institute 1640, ThermoFisher Scientific (ThermoFisher Scientific Gibco™, Germany) supplemented with 20% Fetal Bovine Serum (FBS) (Gibco™, Germany), 1% pen-strep (Penicillin-Streptomycin (Gibco™) and 1% L-Glutamine (200 mM) — Gibco™). Jurkat and K562 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% Penstrep and 1% L-Glutamine.

Tyrosine Kinase Inhibitors and Compounds

The AXL inhibitor R428 (Cat. No. S2841) and MerTk inhibitor UNC2250 (Cat. No. S7342) were purchased from Selleck Chemicals which were diluted in Dimethyl Sulfoxide (DMSO) to a stock solution of 1000x. The working dilutions (1X) were prepared from stock solutions afterwards.

Cytotoxicity/Proliferation Assay

Cytotoxicity was assessed using the MTT assay with the Roche Cell Proliferation Kit (MTT)- Cat No. 11465007001) according to the manufacturer's instructions. Cell viability and growth of cells were additionally evaluated using the Trypan-Blue assay exclusion assay. For the MTT analysis 50,000 cells/well were plated in a 96 well plate. On the basis of MTT results 1.5 μ M inhibitor concentration was chosen for further experiments with Jurkat and K562 while 500nM inhibitor concentration was selected for Molm-13 and Kasumi-1 (Supplementary File. MTT Assay Leukemia).

Western Blotting

Western Blotting was carried out according to widely established protocols. Jurkat and Kasumi-1 cells were treated with MerTk inhibitor UNC2250 for 48 hours in six-well plates at concentrations of 1.5 μ M (Jurkat) and 500nM (Kasumi-1). Similarly, K562 and MOLM-13 cells were treated with Axl inhibitor R428 for 48 hours at concentrations of 1.5 μ M (K562) and 500nM (MOLM-13). Following treatment, cells were collected in separate microcentrifuge tubes by centrifugation at 1200rpm for 5 minutes at 25°C. The cell pellets were washed with PBS and lysed in RIPA buffer (ThermoScientific-89900 containing) supplemented with 1x protease inhibitor (Roche Complete™ Protease Inhibitor Cocktail, Cat. No.: 11836170001) and 1x phosphatase inhibitor (Roche PhosSTOP™, Cat. No.: 4906837001), with volumes adjusted according to pellet size. Lysates were incubated on ice for 2 hours then centrifuged at 14000rpm for 15minutes at 4°C. The resulting supernatants were collected into fresh microcentrifuge tubes. Protein concentration was determined using the Bradford Assay with BSA [37] (ThermoScientific Pierce™ Bovine Serum Albumin Standard, 2mg/ml) as the standard. Absorbance was measured at 595nm using a Thermo Scientific- MULTISKAN Sky microplate reader. Proteins were denatured in lammelli buffer and heated at 95°C for 10 minutes. For SDS-PAGE, 12% resolving and 4% stacking gels were used for proteins below 50kDa while 10% resolving and 7% stacking gels were used for proteins upto 120kDa. Following electrophoresis, the proteins were transferred onto blotting membrane which was blocked in 5% (non-fat dry milk prepared in TBS with 0.1% Tween 20 for 1 hour. After washing membranes were incubated with the secondary antibodies (1:2000 dilution) for 1–2 hours at room temperature with gentle shaking. Protein bands were detected using enhanced chemiluminescence (ECL) and visualized on a Bio-Rad ChemiDoc Imaging System. Primary antibodies used included ERK1/2 (4370S), mTOR (2983S), BCL2 (sc-7382), BAX (sc-7480), Cleaved Caspase 3 (9664S) and B-actin(sc-47778) (Supplementary File. WB-Antibodies).

Striping Procedure

The blot was washed thrice with TBST, stripping buffer (Thermo Fisher Scientific) was added and the blot was washed thrice again. 5% non-fat dry milk was used for blocking for 1 hour. After blocking the blot was washed thrice again with TBST and incubated with the primary antibody overnight.

RT-PCR

Cells were collected after 48-hour treatment with inhibitors and total RNA was extracted using TRizol Reagent (Invitrogen) following manufacturer's protocol. RNA concentration and purity was measured on Nanodrop and cDNA was synthesized from total RNA using OneScript Plus cDNA synthesis kit (Abcam) following manufacturer's instructions. The reverse transcription reaction was performed under the following conditions: 25°C for 2 minutes, 55°C for 20 minutes, 85°C for 5 minutes and held at 10°C until termination. Real Time PCR (q) PCR was carried out using BlasTaq™ 2×qPCR MasterMix (G892). Gene expression analysis was performed for Axin-2, c-Myc and GAPDH the following primers (5'-3'), c-Myc (F): GAAAAGGCCCCCAAGGTAGT: c-Myc (R): AGTTTGTGTTTCAACTGTTC: Axin-2 (F): CTATGTCTTTGCACCAGCCA: Axin-2 (R): TAGAGACACTTGGCCATTGG: GAPDH (F): CATCACCATCTTCCAGGAG: GAPDH (R): GATGATGACCCTTTTGGC. qPCR amplification was performed on the CFX96 Touch Real-Time PCR Detection System using a two-step cycling protocol: initial denaturation at 95°C for 3 minutes, followed by 40cycles of 95°C for 10 seconds and 58°C for 1 minute (Supplementary File. RT-PCR Leukemia).

Densitometric Analysis

Densitometric analysis was carried out using Image Lab (Bio-Rad). Western blot images in*tiff format were imported for analysis. The resulting data tables were exported in excel format. The adjusted lane volume of treated samples was normalized to the untreated control (Supplementary File. Western Blotting-Densitometric Analysis)

Statistical Analysis

Statistical analysis was performed using Graph-pad Prism 8. Paired *t*-tests were used to compare control and experimental groups while unpaired *t*-tests were applied for comparison between different cell lines. A *p* value < 0.05 considered to be statistically significant. All experiments were performed in duplicate (Supplementary File. Statistical Analysis).

RESULTS

AML shows increased sensitivity to TAM receptor inhibitors compared to CML and ALL

MTT, a proliferation assay was carried out to investigate the effect of TAM receptor inhibition on leukemic cell lines representing acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and T-cell acute lymphoblastic leukemia (T-ALL). Jurkat (T-ALL) cells and K562 (CML) cells showed minimal to no response under the same conditions (IC_{50} around 1.5 μ M) (Fig: A, B). In contrast Kasumi-1 (AML) cells and MOLM-13 (AML) cells exhibited strong sensitivity to TAM inhibitors (IC_{50} around 500nM) (Fig: C, D). These findings suggest that AML cell lines are more susceptible to TAM inhibition compared to CML and T-ALL cell lines. These findings suggest that AML cell lines are more susceptible to TAM inhibition compared to CML and T-ALL cell lines.

Targeting TAM receptors disrupts canonical WNT signaling pathway

Multiple signaling pathways, including RAS/MAPK/ERK, Wnt and PI3K/AKT/mTOR pathways are reported to be constitutively active in leukemia thereby driving oncogenesis. Following the proliferation assays, we next aimed to evaluate the effect of targeted TAM receptor inhibitions on downstream targets of these oncogenic pathways. Expression analysis revealed that the Wnt component Axin-2 was significantly upregulated in AML representative cell lines namely Kasumi-1 and MOLM-13 cells while it was downregulated in CML and ALL representative cell lines, K562 and Jurkat (Fig. 2A-D). Similarly, c-Myc expression was significantly upregulated in MOLM-13 cells compared to the other cell lines analyzed (Fig. 2E-H) (Supplementary File Statistical Analysis: Table S1 & 2).

AML exhibits elevated expression of canonical WNT signaling pathway effectors compared to CML and ALL

To investigate the involvement of WNT signaling pathway in apoptosis induction, expression analysis was performed across all representative leukemic cell lines following inhibition of TAM kinase receptors, which are known to be overexpressed in these models as reported previously [38–41]. Expression level of WNT signaling pathway components were found to be elevated in AML cell lines compared to CML and ALL (Supplementary File Statistical Analysis Table S3 & S4).

Figure 3 Comparative analysis of WNT signaling components after targeted treatment (A) Axin-2 between Jurkat and Kasumi-1. (B) Axin-2 expression in K562 compared with Molm-13. (C) c-Myc expression in Jurkat compared to Kasumi-1. (D) c-Myc expression in K562 compared to Molm-13. The p value measured with unpaired t tests (comparison between different cell lines) is represented as *p < 0.05, **p < 0.001, ***p < 0.0001. A p value > 0.05 is shown as not significant (ns) in the figure

TAM kinase receptor inhibition induces differential expression of ERK1/2 and mTOR in leukemic cell lines

To further elucidate the role of oncogenic signaling towards leukemogenesis, key downstream components of RAS/MAPK and PI3K/AKT pathways were analyzed by western blotting. The cell lines

were treated based on IC₅₀ values derived from the proliferation assay. Band intensities were quantified using Image Lab software and the adjusted lane volume were normalized to the untreated control. The resulted values are plotted as bar charts (Figure: 4A-H). ERK1/2 was downregulated in treated samples of Jurkat and K562 cells compared to the untreated control (Fig. 4A, C), whereas it was upregulated in Kasumi-1 following treatment (Fig. 4.B). In MOLM-13 cells ERK1/2

showed a slight decrease upon treatment with the kinase inhibitor relative to the untreated control (Fig. 4D). mTOR expression was downregulated in all cell lines except Kasumi-1 (Figure: 4 E-H).

Figure 4 Densitometric analysis of ERK1/2 and mTOR signaling components in leukemic cell lines. (A) ERK1/2 expression in control vs treated Jurkat. (B) ERK1/2 expression in control vs treated Kasumi-1. (C) ERK1/2 expression in control vs treated K562. (D) ERK1/2 expression in control vs treated Molm-13. (E) mTOR expression in control vs treated Jurkat (F) mTOR expression in control vs treated Kasumi-1 (G) mTOR expression in control vs treated K562 (H) mTOR expression in control vs treated Molm-13

ERK1/2 and mTOR modulation upon TAM Inhibitors treatment indicates involvement of RAS/MAPK and PI3K/AKT signaling

Despite elevated ERK1/2 and mTOR levels, Kasumi-1 cells exhibit proliferation inhibition upon treatment. Similarly, in MOLM-13 cells, the concurrent upregulation of BCL2 and Axin-2 (Axin-2/BCL2 axis) is associated with proliferation inhibition (Figure:5 A-C).

Densitometric analysis of total protein expression of intrinsic apoptotic pathway components

The observed inhibition of proliferation despite elevated oncogenic signaling components prompted further investigation of the intrinsic apoptotic pathway to explore these contrasting findings. Densitometric analysis of western blots revealed that the intrinsic apoptotic response depends on WNT signaling pathway. Specifically, upregulation of Axin-2 is associated with downregulation of BCL2 thereby inducing apoptosis in Kasumi-1 cells. In contrast, downregulation of Axin-2 coupled with increased BCL2 levels appears to inhibit apoptosis in Jurkat and K562 cells. In MOLM-13 cells a relatively higher expression of Axin-2 compared to BCL2 is associated with proliferation inhibition (Figure: 6).

The Axin-2-BCL2 axis modulates apoptosis upon targeting TAM kinase Receptors in leukemia

Axin-2 appears to play a key role in apoptosis induction. In K562 and Jurkat cells, downregulation of Axin-2 is associated with upregulation of BCL2 following Axl and MerTk inhibition, suggesting suppression of apoptosis. In contrast, in Kasumi-1 cells upregulation of Axin-2 upon MerTk targeting

correlates with decreased BCL2 expression, indicative of enhanced apoptotic activity. In MOLM-13 cells, Axl inhibition likely interferes with FLT3-ITD driven signaling, contributing to cell cycle arrest. Overall, the Axin-2/BCL2 axis emerges as a potential therapeutic target for sensitizing leukemic cells to apoptosis.

Table 1
The reported data collected before targeting TAM Kinase Receptors

Type of Leukemia	Signaling Pathway Effectors	Type of TAM Kinase Receptor Overactive	Effect on BCL2 before Targeting TAM Kinase Receptors
AML (MOLM-13)	ERK1/2 & mTOR active [42]	AXL [43]	High BCL2
AML (Kasumi-1)	mTOR active [44]	MerTk [39, 45]	High BCL2
CML (K562)	ERK1/2, mTOR & WNT active[46]	AXL [47]	High BCL2
ALL (Jurkat)	ERK1/2, mTOR active[48]	MerTk[38]	High BCL2

Table 2: Summary of results based on our experimental data after targeting TAM Kinase Receptors

Type of Leukemia	Signaling Pathway Effector	Effect on BCL2 after targeting TAM Kinase Receptors
AML (MOLM-13)	ERK1/2 mTOR WNT ↓	High
AML (Kasumi-1)	ERK1/2 mTOR WNT ↑	Low
CML (K562)	ERK1/2 mTOR WNT ↓	High
ALL (Jurkat)	ERK1/2 mTOR WNT ↓	BCL2=BAX

DISCUSSION

Intrinsic apoptotic signaling has been reported to depend on oncogenic signaling effectors in nearly all types of leukemia. The aim of this study was to investigate the effect of targeting TAM kinase receptors upon apoptosis induction via the intrinsic apoptotic pathway. TAM Kinase receptors are overexpressed in

most types of leukemia regulating cell death. The BCL2 oncogene protects cells against apoptosis promoting therapy resistance in leukemia particularly in acute myeloid leukemia (AML)[49–51]. In addition, BCL2 and mTOR exhibit functional crosstalk, which has been successfully targeted in other malignancies such as colorectal cancer and diffuse large B- cell lymphoma (DLBCL) [52–54]. mTOR regulates the intrinsic apoptotic pathway through BCL2[55]. ERK1/2 positively regulates BCL2 to promote cell survival [56]. Most of the studies have reported that simultaneous targeting of BCL2 with either mTOR or BCL2 or ERK1/2 sensitizes leukemic cells to apoptosis. The Axl receptor is over expressed in MOLM-13 and K562 while MerTk predominantly expressed in Kasumi-1 and Jurkat cells [15]. When targeted with TAM inhibitors these cell lines exhibit markedly different responses. Severe proliferation inhibition is observed in Kasumi-1 and MOLM-13 compared to Jurkat and K562 cells when treated with TAM kinase inhibitor (Fig. 1). Furthermore, WNT signaling components are elevated in AML cell lines while they are downregulated in Jurkat and K562 cells (Fig. 2). Likewise, mTOR and ERK1/2 expression level are comparatively higher in AML cell lines compared to CML and ALL cell lines (Fig. 2). According to previous studies, oncogenic signaling operates in close coordination with mitochondrial signaling pathways thereby halting apoptosis. Our findings indicate that targeting TAM kinase receptors with specific inhibitors strengthens this crosstalk between oncogenic effectors and mitochondrial signaling components (Fig. 3). Despite elevated ERK1/2 and mTOR levels, apoptosis is induced in Kasumi-1 cells upon targeting MerTk receptor. In addition, BAX is upregulated in Kasumi-1 cells, while BCL2 is elevated in MOLM-13(Fig. 3) both of which exhibit proliferation inhibition (Fig. 1). In MOLM-13, proliferation inhibition appears to result from an imbalance in the Axin-2/BCL2 axis. Overall, the results indicate that low Axin-2 and high BCL2 may contribute to apoptosis resistance in CML and ALL representative cell lines despite downregulation of other oncogenic signaling components (Figs. 3 & 7). Axin-2 overexpression has been reported to enhance apoptosis whereas its silencing reduces apoptotic activity [31]. In the current study, components of the canonical WNT, PI3K/AKT and RAS/MAPK signaling pathways were elevated in AML in comparison to CML and ALL (Fig. 2–5). Interestingly, despite this elevated expression, proliferation inhibition was evident in AML, whereas CML and ALL cell lines showed comparatively lower inhibition and reduced pathways signaling components (Fig. 1–5). To find the reason behind such behavior of leukemic cell lines, intrinsic apoptotic pathway was further analyzed and compared across the cell lines (Fig. 6 & 7). Oncogenic signaling pathways interact with the mitochondrial pathway to regulate apoptosis[57]. TAM receptors are reported to be aberrantly expressed in leukemia and contribute to therapy resistance and relapse[14]. Notably, different leukemia subtypes exhibit distinct response to TAM kinase inhibitors, at comparable concentration, proliferation inhibition is more pronounced in AML cell lines than in CML and ALL cell lines (Fig. 1). Analysis of WNT signaling components in treated versus untreated samples revealed a significant upregulation of Axin-2 in AML cell lines (Fig. 2). The difference in c-Myc expression between untreated and treated samples was not significant in Jurkat, Kasumi-1 and K562, however Molm-13 showed a significance in expression (Fig. 2). Comparative analysis of AML, CML and ALL cell lines with respect to canonical WNT signaling revealed marked difference between Jurkat and Kasumi-1 as well as between K562 and Molm-13 (Fig. 3). Axin-2 was overexpressed in Kasumi-1 and MOLM-13 and the observed induction of apoptosis in Kasumi-1 (Fig. 7) is consistent with previous findings [31]. Normally, ERK is known to regulate the G1-S phase

transition under physiological conditions; however, its overexpression has been associated with cell cycle arrest[58, 59]. Proliferation inhibition in MOLM-13 appears to be mediated through the Axin-2/BCL2/c-Myc axis (Fig. 4D & Fig. 7). A possible explanation for the induction of cell cycle arrest in MOLM-13, despite elevated BCL2 and Axin-2 levels, is the interaction of c-Myc with BCL2 which may have modulated apoptotic signaling [36]. Additionally, ERK1/2 and mTOR effectors of the RAS/MAPK and PI3K/AKT pathways are also influenced by TAM kinase receptor inhibition. Specifically, ERK1/2 and mTOR are upregulated relative to untreated control in Kasumi-1, whereas they are downregulated in the other representative cell lines (Figure.4). Following targeted treatment, ERK1/2 was upregulated in Kasumi-1 and MOLM-13 compared to the other cell lines (Fig. 5). Densitometric analysis of the intrinsic apoptotic pathway indicated that BCL2 expression correlates with Axin-2 level, indicating a potential functional link between these proteins (Fig. 6). Comparative analysis of western blot and real time PCR data further showed that Kasumi-1 exhibits high Axin-2 and low BCL2 relative to Jurkat while MOLM-13 shows elevated expression of Axin-2, c-Myc and BCL2 levels compared to K562 (Fig. 7). These findings are consistent with previous reports and support the association between Axin-2 and BCL2 expression. Overall, the Axin-2/BCL2 axis emerges as a prominent feature in this study, highlighting its potential as a possible therapeutic target.

Declarations

Ethical Approval: The Institutional Review Board (IRB No. 02-2026-IRB-A-143/143) reviewed and approved by the National University of Sciences and Technology, NUST in accordance with the standards mentioned in the WMA declaration of Helsinki'1964 and thereafter.

Declaration of generative AI use: The authors have used <https://elicit.com/> for in depth knowledge and research related information.

Consent for Publication: All authors read and approved the final draft for publication.

Availability of Data: Data is available on request to corresponding author.

Competing Interests: No competing interest

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Author Contributions: Conceptualization, SN, AAM and MA; methodology, SN. and AAM; validation, SN; formal analysis, S.N; investigation, S.N; resources AAM and MA; writing original draft preparation, S.N; writing, review and editing, SN. HMW, AAM and MA; visualization, S.N.; supervision, AAM, HMW and MA. All authors have read and agreed to the published version of the manuscript.

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Figures

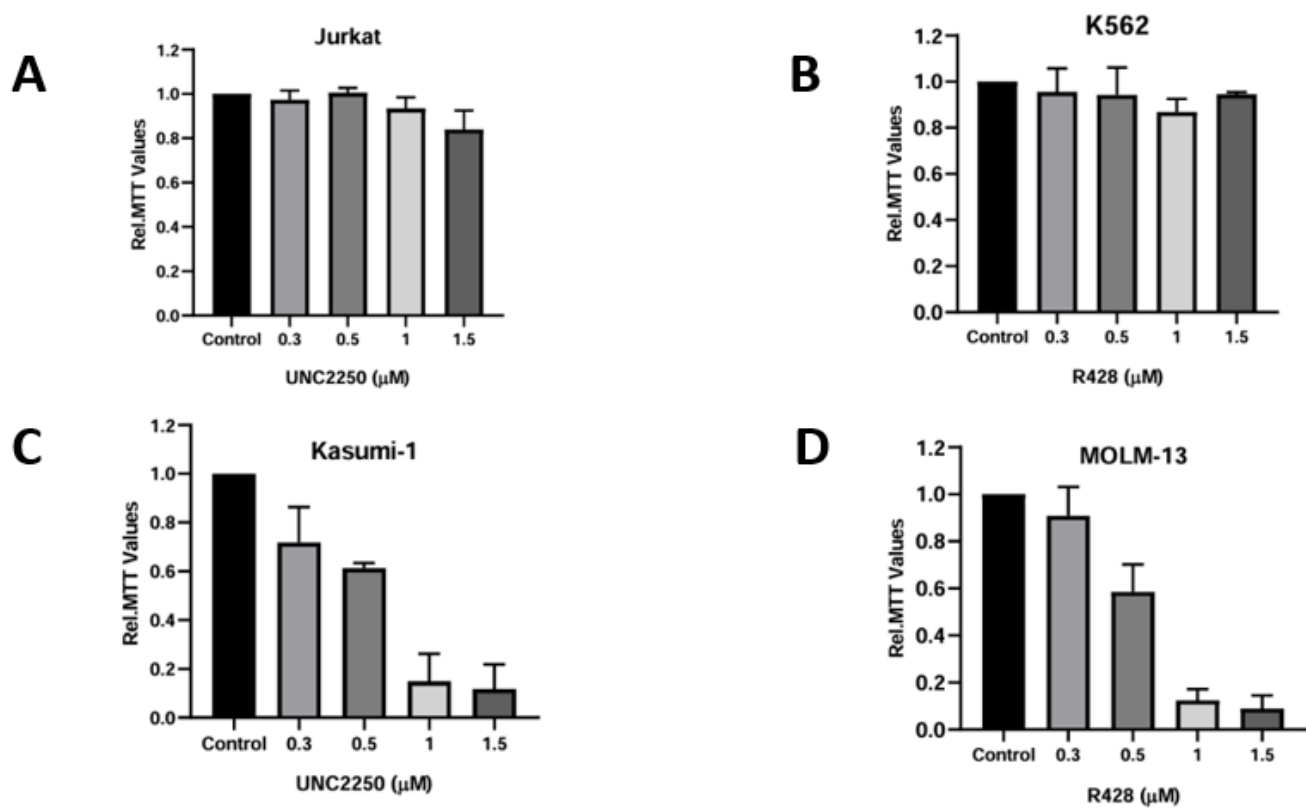


Figure 1

MTT Assay with TAM kinase Inhibitors was carried out on leukemic cell lines namely: Jurkat, (A) K562 (B), Kasumi-1 (C) and Molm-13 (D). AML cell lines (C, D) reflect potential proliferation inhibition in comparison to CML (K562) and ALL (Jurkat) cell lines at similar concentrations. The mean +/- SD of two experiments are given

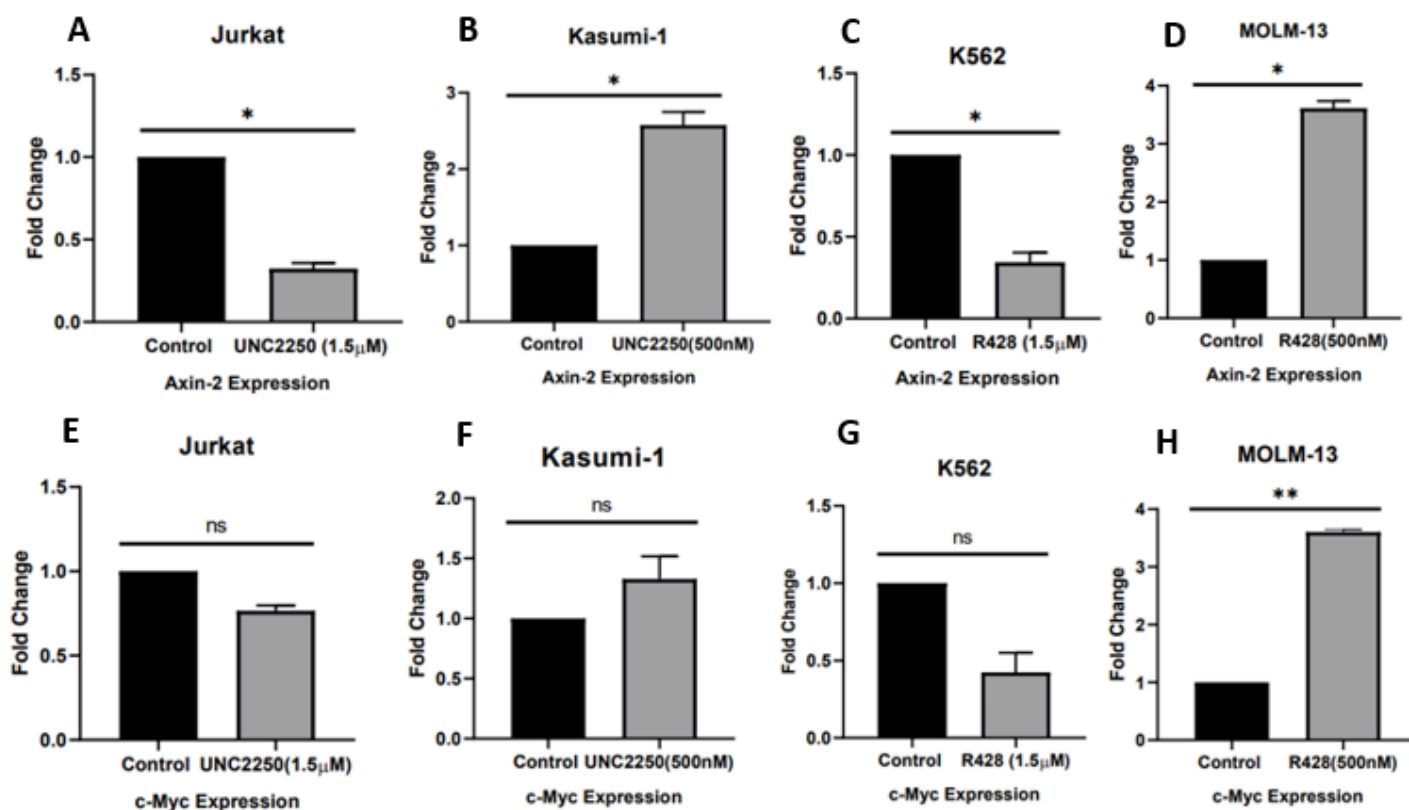


Figure 2

Expression analysis of Canonical WNT signaling components. (A) Axin-2 expression in Control Vs MerTk inhibited (IC₅₀=1.5 μ M) samples of Jurkat cell line (B) Axin-2 expression in Control Vs MerTk inhibited (IC₅₀=500nM) samples of Kasumi-1. (C) Axin-2 expression in control vs R428 treated (IC₅₀=1.5 μ M) K562 samples. (D) Axin-2 expression in control vs R428 treated (IC₅₀=500nM) Molm-13 samples. (E) c-Myc expression in control vs UNC2250 treated (IC₅₀=1.5 μ M) Jurkat samples. (F) c-Myc expression in control Vs UNC2250 treated (IC₅₀=500nM) kasumi-1 samples. (G) c-Myc expression in control vs R428 treated (IC₅₀=1.5 μ M) K562 samples. (H) c-Myc expression in control vs R428 treated (IC₅₀=500nM) Molm-13 samples. The p value measured with paired t tests (comparison between control and individual concentrations) is represented as *p<0.05, **p<0.001, ***p<0.0001. A p value >0.05 is shown as not significant (ns) in the figure

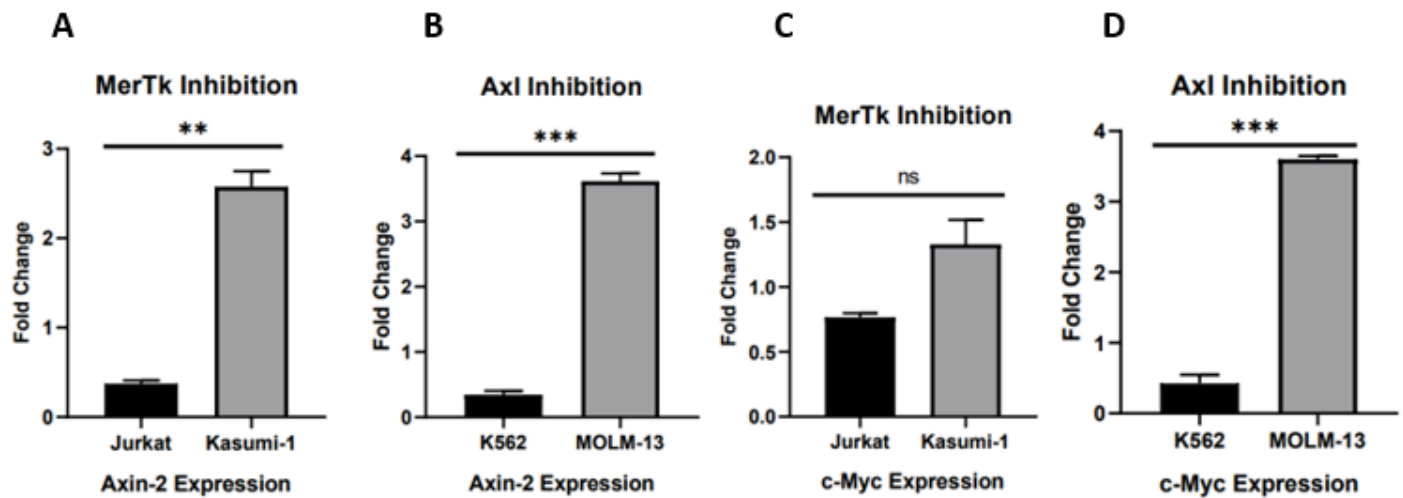


Figure 3

Comparative analysis of WNT signaling components after targeted treatment (A) Axin-2 between Jurkat and Kasumi-1. (B) Axin-2 expression in K562 compared with Molm-13. (C) c-Myc expression in Jurkat compared to Kasumi-1. (D) c-Myc expression in K562 compared to Molm-13. The p value measured with unpaired t tests (comparison between different cell lines) is represented as * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. A p value > 0.05 is shown as not significant (ns) in the figure

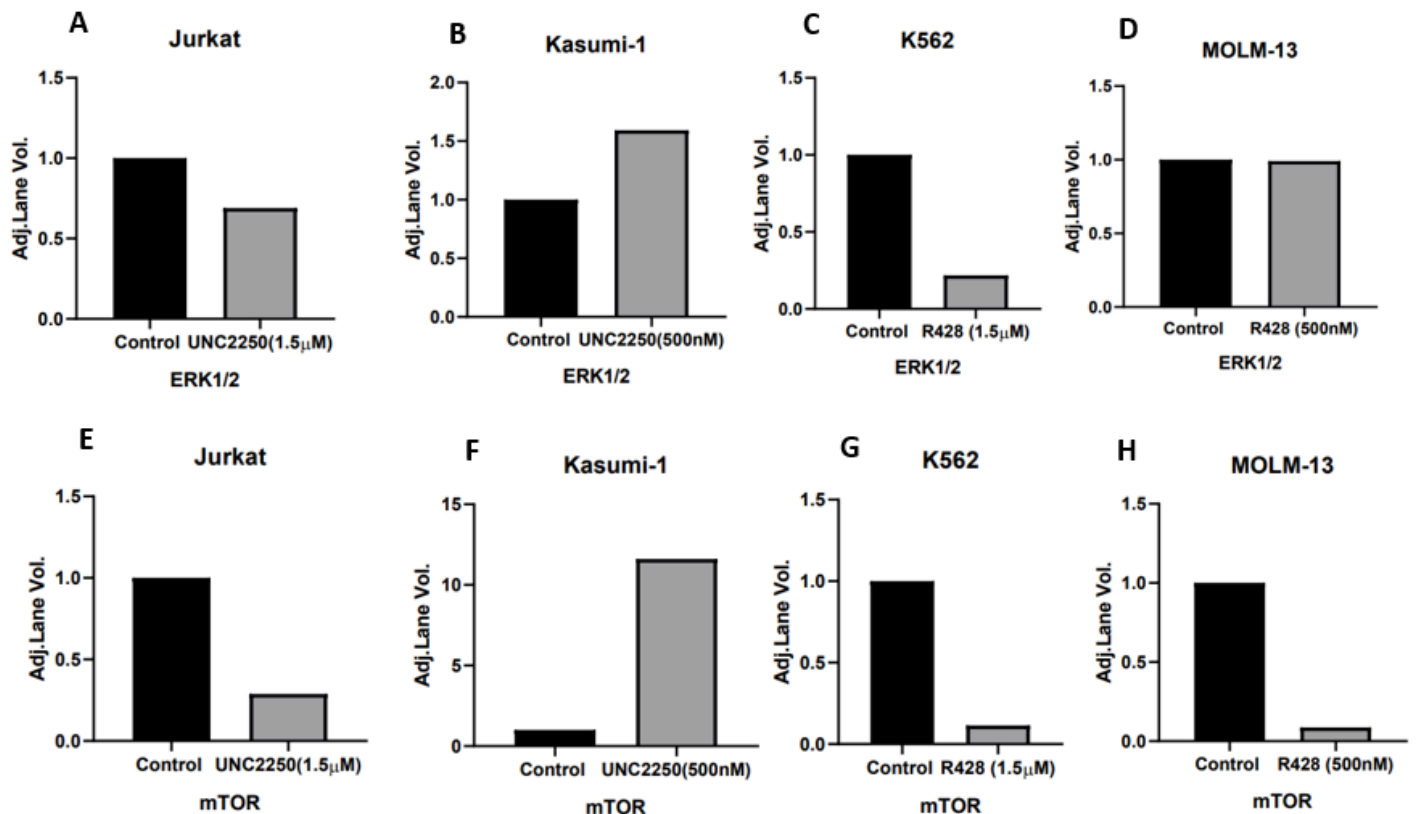


Figure 4

Densitometric analysis of ERK1/2 and mTOR signaling components in leukemic cell lines. (A) ERK1/2 expression in control vs treated Jurkat. (B) ERK1/2 expression in control vs treated Kasumi-1. (C) ERK1/2 expression in control vs treated K562. (D) ERK1/2 expression in control vs treated Molm-13. (E) mTOR expression in control vs treated Jurkat (F) mTOR expression in control vs treated Kasumi-1 (G) mTOR expression in control vs treated K562 (H) mTOR expression in control vs treated Molm-13

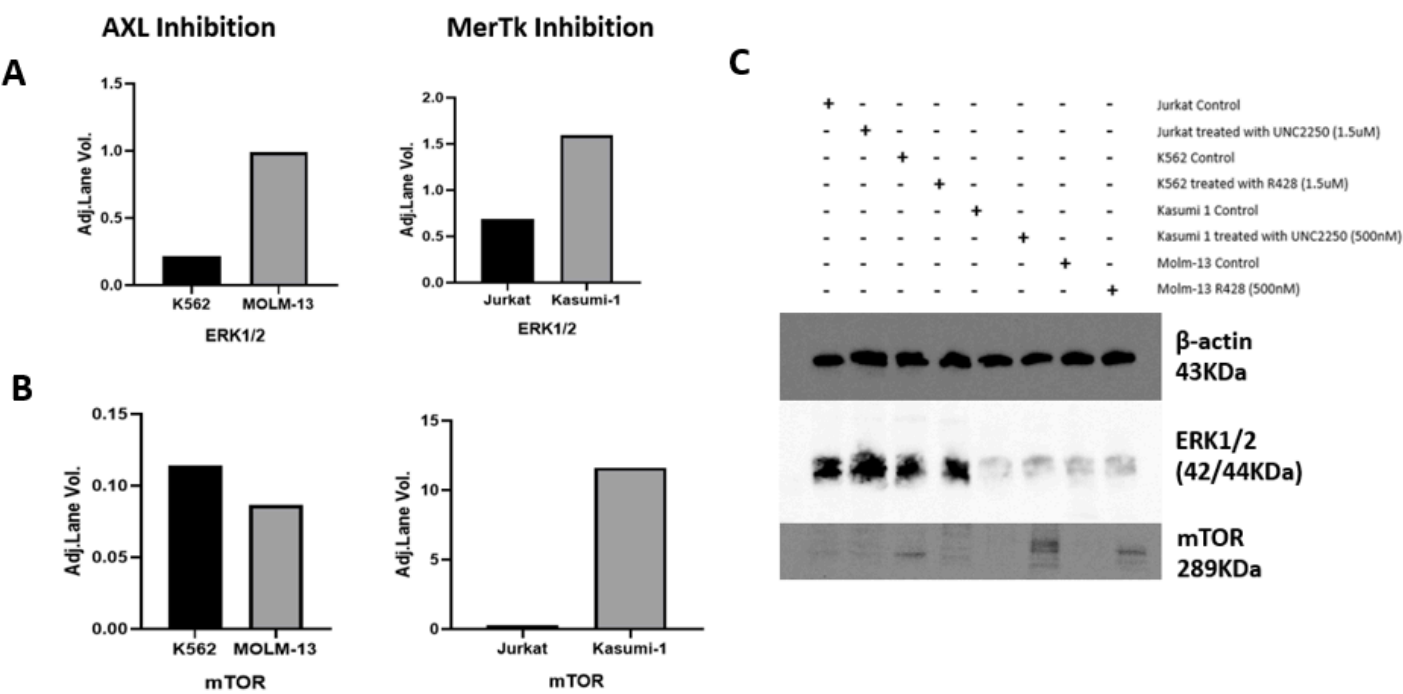


Figure 5

Comparison of expression of oncogenic signaling components in leukemic cell lines upon targeting TAM Kinase receptors. (A) ERK1/2 is much low in volume in K562 in comparison to Molm-13 and same is the case with Jurkat aasumiumi-1 where expression of ERK1/2 is much higher in Kasumi-1. (B) Although the volume of mTOR is low in both K562 and Molm-13 yet K562 is slightly better than Molm-13. mTOR expression in Kasumi-1 is very high in comparison to Jurkat indicating potential PI3K/AKT involvement. (C) Western Blots of treated and untreated samples

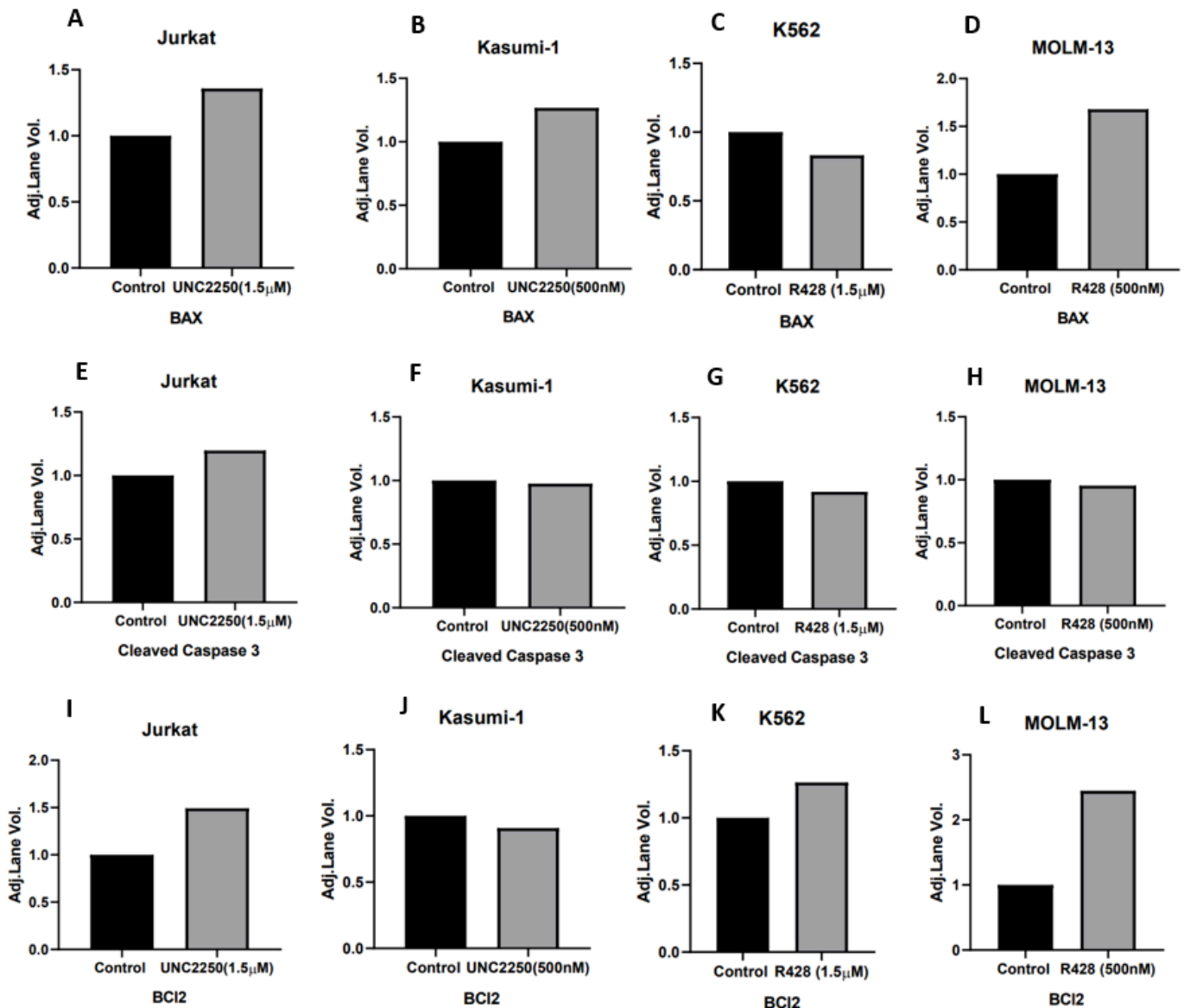


Figure 6

Densitometric expression analysis of intrinsic apoptotic components. (A, E, I) elevated BAX and Cleaved Caspase 3 cannot induce apoptosis due to a high Bcl2 in Jurkat cell line. (B, F, J) A High BAX and low Bcl2 induces apoptosis in Kasumi-1. (C, G, K) A low BAX, Low cleaved caspase 3 and High Bcl2 prevents apoptosis induction in K562. (D, H, L) A high Bcl2 in comparison to BAX and Cleaved Caspase3 prevents death by intrinsic apoptotic pathway so, the proliferation inhibition is due to cell cycle arrest

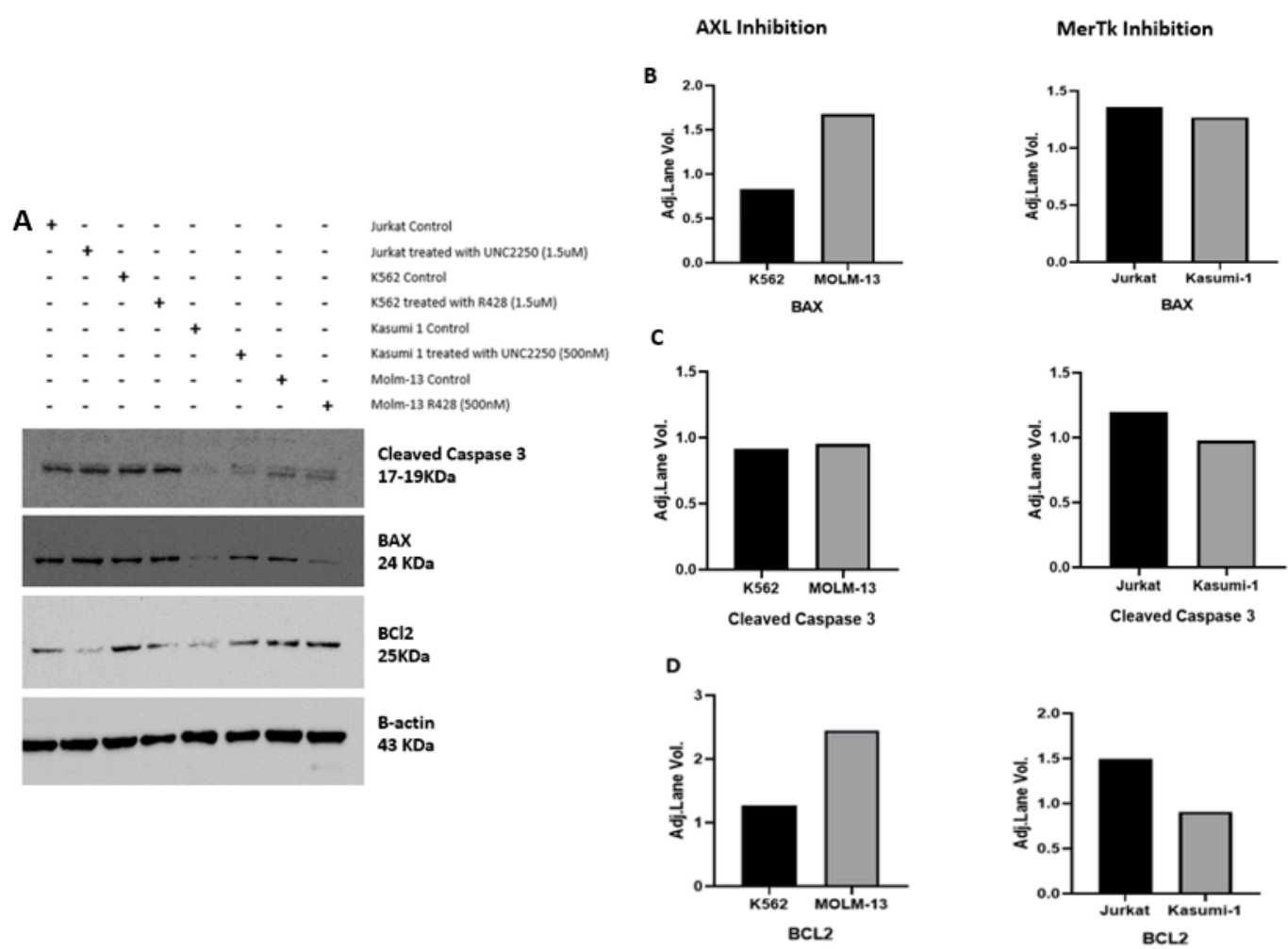


Figure 7

Comparative Translational analysis of components of mitochondrial apoptotic pathway. (A) Western Blots of treated and untreated samples. (B) Comparison of BAX in treated samples of K562 and Molm-13 and Jurkat and Kasumi-1. (C) Comparison of Cleaved caspase 3 among treated samples of K562 and Molm-13 and Jurkat and Kasumi-1. (D) Comparison of BCL2 among treated samples of K562 and Molm-13 and Jurkat and Kasumi-1

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