Regulation of lncRNAs upon dual inhibition of PI3K/Akt/mTOR in leukaemic cells

ABSTRACT

Leukaemia is the tenth leading cause of death worldwide. Small-molecule inhibitors have recently been used to treat leukaemia. This study assessed the effectiveness of different PI3K/Akt/mTOR pathway inhibitors such as everolimus, PP242, and PKI402 on the acute T-lymphoblastic leukaemia (T-ALL) Jurkat cell line. Furthermore, qPCR experiments were performed to test the effect of these drugs on the expression of different long non-coding RNAs (lncRNAs). In proliferation experiments, PKI402 was 65% effective against cell viability; this efficacy was the greatest of the three molecules. PKI402 also inhibited phosphorylation intensity by two-thirds, as measured by pAkt and pS6. Everolimus showed the same effect on cells only when pS6 was measured. All three drugs exhibited significant efficacies of ~ 50-60% arrest in cell cycle experiments. When everolimus or PKI402 were used to treat the cells, the cells arrested in G1 phase. However, PP242 arrested the cells in S phase. Long non-coding RNAs play an important role in genome regulation, and they are commonly altered in cancers. We screened the expression of 96 cancer-associated lncRNAs before and after the PI3K/Akt/mTOR pathway inhibition. We found that the T-ALL cell line responded well to target therapies, and that lncRNA expression levels differed between single and dual target therapy inhibitors. These results indicate that an mTORC1 inhibitor, either as a single specific target or in combination with PI3K inhibitors, may be a viable treatment for T-ALL. However, dual-specificity inhibitors such as PKI402 also show promise against T-ALL. In addition, the role of lncRNAs in drug resistance in this cell line needs further investigation to identify opportune treatment targets.

Keywords: Leukaemia, target therapy, lncRNA, everolimus, PKI402
1. INTRODUCTION

The National Cancer Institute defines leukaemia—cancer of the white blood cells (WBCs) \(^{1}\)—as "cancer that starts in blood-forming tissue, such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the bloodstream." In leukaemia patients, the bone marrow (BM) loses its ability to produce normal WBCs, and hence, its main function in fighting infections \(^{1,2}\). Leukaemia is the tenth leading cause of death worldwide, with a mortality rate of about 3.2% in both sexes \(^{3}\).

Due to differences in the development and rapidity of disease onset, leukaemias are divided into two major groups, acute or chronic, which are further divided into either myeloid or lymphoid. The classification of these types is based on the specific BM cell lineages that are affected. Consequently, there are four subtypes of leukaemia: acute lymphoid leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphoid leukaemia (CLL), and chronic myeloid leukaemia (CML) \(^{1}\). Acute lymphoid leukaemia is the most common leukaemia in children, whereas adults are mostly affected by AML and CLL \(^{2}\).

Acute leukaemia can develop, and its prognosis can deteriorate, rapidly. The number of abnormal and non-functional WBCs increases rapidly along with an increase in the number of leukaemic blast cells in the BM, and a subsequent decrease in the number of normal blood cells \(^{1}\). On the other hand, chronic leukaemia develops slowly, and leukaemic WBCs can function similarly to normal WBCs. Left untreated, however, the leukaemic WBCs will accumulate in the BM in excess of normal blood cells, potentially causing complete loss of function \(^{1}\).

Leukaemia treatment approaches vary according to the type of leukaemia and the patient’s general health and age. Current therapies include chemotherapy, radiation therapy, antibiotics, stem cell transplantation, and targeted therapy \(^{4}\). Targeted therapies, also called "molecularly targeted therapies", "molecularly targeted drugs" or "precision medicines", comprise drug molecules that interfere with a target molecule or "specific molecule" \(^{1}\). These drugs may block the metastasis and growth of cancer cells, but not those of normal cells, upon
interference with the target molecule, which is often involved in different cancer cell functions such as progression, growth, and metastasis [1]. Targeted therapy development requires identification of the most compatible target molecule that has a role in cancer cell growth and survival. Target identification involves comparing normal and cancer cells from genomic, proteomic, or transcriptomic perspectives [1].

Although targeted cancer therapy has less severe and toxic side effects than those elicited by standard chemotherapy because of its different mechanism of action, it has some general side effects [1,5]. These include diarrhoea, fatigue, and acne-form dermatitis [5]. Targeted therapies include hormone therapies, apoptosis inducers, angiogenesis inhibitors, immunotherapies, gene expression modulators, toxin delivery molecules and signal transduction inhibitors [1]. The most intensely studied drugs are signal transduction inhibitors of the phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (mTOR) signalling pathways. These pathways regulate and control important cellular processes such as growth, survival, and apoptosis. PI3Ks are members of the lipid kinase family [6,7], which comprises enzymes that generate 3-phosphorylated inositol lipids and promote cell membrane responses to specific effectors [7,8]. PI3K phosphorylates, and hence activates, Akt [9]. Akt, or protein kinase B, is a serine/threonine protein kinase [7,9]. The PI3K/Akt pathway regulates cell survival during stress. It has a crucial role in regulating tumour cell responses to intrinsically stressful environments, such as low nutrient and oxygen supply and low pH [10]. Akt can be activated by mTOR [9], a serine/threonine/protein kinase that controls cell growth, cell proliferation, cell motility, and angiogenesis [10]. mTOR comprises two biochemical complexes: mTORC1 and mTORC2 [11]. mTORC1 controls nutrient- and growth factor-induced cell growth by regulating the S6K1 and 4E-BP1 molecules. On the other hand, mTORC2 regulates cell proliferation and survival by phosphorylating and thus activating Akt at Ser473 [11].

Several studies have indicated an important relationship between the PI3K/Akt/mTOR pathways and cancer pathogenesis, implicating them as promising cancer therapy targets [7,12]. Targeting approaches could follow one of three mechanisms: (1) the targeting of Akt- and PI3K-activating kinases, (2) the direct
targeting of PI3K or Akt or (3) the targeting of mTOR, which is a downstream effector of Akt [7].

Long non-coding RNA (also referred to as large RNA, macroRNA, and long intergenic ncRNA) is a topic of recent interest in cancer research. These RNAs are mRNA-like transcripts. Their lengths vary between 200 nucleotides and 100 kilobases (kb). They lack open reading frames of meaningful length [13,14,15]. Originally, they were discovered using large-scale sequencing for mouse’s full-length cDNA libraries [16]. They are scattered between coding and non-coding transcripts [17,18,19].

Long ncRNAs have a broad spectrum of molecular and cellular functions. They consist of a heterogeneous group of RNA molecules; thus, they implement diverse modes of action [20]. In cancer literature, they are emerging as major players, with possible roles in the oncogenic and tumour suppressor pathways [15]. Cellular functions remain unknown for many recently discovered IncRNAs. Questions remain as to which of these IncRNAs have significant functions, and which are the “noise” of background transcription [21,22,23,24].

The present study investigated the effect of targeting the PI3K/Akt/mTOR signalling pathways on expression levels of a range of IncRNAs in leukaemic (Jurkat) cells using three different drugs as targeted therapies: everolimus (RAD001), phosphokinase inhibitor 402 (PKI402), and PP242. Everolimus is a first-generation rapamycin analogue (rapalog); first-generation rapalogs inhibit mTORC1 only [25]. The single molecule inhibitor PP242 is a second-generation mTOR inhibitor, which is selective for mTORC1/2 [25]. Phosphokinase inhibitor 402 is also a second-generation mTOR inhibitor, which acts as a dual-specificity PI3K/mTOR inhibitor that targets PI3K in addition to both mTORC1 and mTORC2 [26].
2. MATERIALS AND METHODS

2.1 Cell culture

For standing culturing, T-ALL Jurkat cells (CLS, Eppelheim, Germany) were cultured in Iscove’s Modified Dulbecco’s Media (IMDM; Lonza, Basel, Switzerland) supplemented with 10-20% foetal bovine serum (FBS; Lonza) and 1% penicillin-streptomycin (#17-602E; Lonza). The cells were incubated at 37 °C under a 5% CO₂ atmosphere.

2.2 Phosphorylation experiments

The cells were counted and plated in a 6-well plate at 0.5 × 10⁶ cells/well. Everolimus, PP242, and PKI402 (#S1120, #S2218, and #S2739, respectively; Selleckchem, Munich, Germany) were each added to a separate well at final concentrations of 1 μM; the remaining three wells were left untreated (negative control cells). The plated cells were incubated for 24 h at 37 °C under a 5% CO₂ atmosphere. Subsequently, the cells were collected, centrifuged at 4 °C for 5 min at 400 × g, washed, and transferred to Eppendorf tubes. For staining, the FlowCellect PI3K-mTOR Signalling Cascade kit (#FCCS025210; Merck Millipore) was used. Briefly, 250 µL of 1 × fixation buffer was added to the cells, and the mixture was incubated on ice in the dark for 20 min. Subsequently, the cells were centrifuged at 4 °C for 5 min at 400 × g, and permeabilised on ice in the dark for 20 min with 250 µL of 1× permeabilisation buffer. The cells were centrifuged at 4 °C for 5 min at 400 × g and stained for immunoflow cytometry. All treated cells were stained with an anti-phospho-Akt1/PKBα (Ser-473)-Alexa Fluor488-conjugated monoclonal antibody (α-pAkt) and an anti-phospho-ribosomal protein S6 (Ser-235)-PerCP-conjugated monoclonal antibody (α-pS6). The four untreated samples were also stained with α-pAkt + α-pS6, α-pAkt only, α-pS6 only, or no stain. All antibodies were added at a final 0.5 × concentration. All cells were incubated on ice in the dark for 1 h and subsequently washed, by centrifuging at 400 × g for 5 min at 4 °C and resuspending in 1 mL of assay buffer. Washing was followed with flow cytometry analysis (BD FACSDiva, Becton Dickinson).
2.3 Cell cycle and proliferation experiments

The cells were counted and plated in a 6-well plate at 0.5 × 10^6 cells/well. Everolimus, PP242, PKI402, adriamycin (Adrim; Dabur Pharmaceuticals Ltd., Mumbai, India), and colcemid (#15210-040; Life Technologies) were added at final concentrations of 1 µM to separate wells, and the remaining well was left untreated (negative control cells). The plate was incubated for 48 h at 37 °C under a 5% CO₂ atmosphere. The cells were counted to assess viability, centrifuged at 1100 rpm for 10 min at 4 °C, washed twice with culture media, and resuspended in 5 mL of phosphate-buffered saline (PBS; #17-516F; Lonza). The cells were fixed by adding dropwise 3 mL of 100% ethanol under medium-speed vortexing, and storing at -20 °C for one week. Prior to analysis, the cells were twice washed with PBS and stained with 0.5 mL of propidium iodide combined with RNase (#P4170; Sigma). All samples were incubated at 4 °C in the dark for three h, and subsequently analysed using flow cytometry. The results were analysed using the Modfit LT software (Verity Software House, Topsham, ME, USA).

2.4 RNA extraction and qPCR experiments

The cells were counted and plated in a 6-well plate at 0.5 × 10^6 cells/well. Everolimus, PP242, and PKI402 were added to separate wells at final concentrations of 1 µM each, and the remaining three wells were left untreated (negative control cells). The plated cells were incubated for 48 h at 37 °C under a 5% CO₂ atmosphere. RNA was extracted from the treated and untreated cell lines using mirVana miRNA Isolation Kit (#AM1560; ThermoFisher Scientific). The extracted RNA was converted to cDNA using the standard protocol for the GoScript Reverse Transcription System (#A5000; Promega). Finally, the human and mouse LncProfilers™ qPCR Array Kits (System bioscience) were used to perform the IncRNA 96-well plate assay (Supplementary Figure 1); the SYBR® Green PCR Master Mix and SYBR Green RT-PCR Reagents Kit (ThermoFisher # 4309155) were used to perform the qPCR experiments using the primers in Table 1.
2.5 Transfection of small interfering RNA (siRNA)

To suppress expression of Zfhx2as in cancer cells, custom-designed siRNA targeting Zfhx2as and control scramble were obtained from Dharmacon, UK. The siRNAs were transfected into cells using Lipofectamine® RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions.

Table 1: Primer sequences for lncRNAs

<table>
<thead>
<tr>
<th>IncRNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANRIL</td>
<td>TTGAACTAAAGCCGCTCCG</td>
<td>TGGTGCCCCAGAAAACAGAAG</td>
</tr>
<tr>
<td>NEAT1</td>
<td>GGTCTGTGTGGAAGGAGGAA</td>
<td>GCTGGCATGGACAAGTTGAA</td>
</tr>
<tr>
<td>RNCR3</td>
<td>AGTCAGTGCTGGGCTTTATT</td>
<td>AAGAGGCTCGTCATGTGGA</td>
</tr>
<tr>
<td>UCA1</td>
<td>AAAGCTGCCCTCTCTATC</td>
<td>CAGGTGGATCTCTTCCAGGA</td>
</tr>
<tr>
<td>HULC</td>
<td>GGGGTGGAACTCATGATGGA</td>
<td>TGGAGGTTGAAATGTCACG</td>
</tr>
<tr>
<td>Zfhx2as</td>
<td>AGATCCCCTTGTCTGGTG</td>
<td>AGGCAGTGTCAGGATCTTC</td>
</tr>
<tr>
<td>HOXA3as</td>
<td>AGAAACCCACGCTTTTCCC</td>
<td>CTGCTCCAAACTTCTGCAC</td>
</tr>
<tr>
<td>RPL11 (+ve control)</td>
<td>ATCCTTTGGGATCCGGAGAA</td>
<td>ACCACATAGAAGTCACGGCC</td>
</tr>
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</table>
3. RESULTS

3.1 Phosphokinase inhibitor 402 is more effective at killing leukaemic cells and arresting their cell cycles than single molecule inhibitors.

**Figure 1:** Mean of three proliferation experiments illustrating cell counting.

**Figure 2:** Mean of three cell cycle experiments treated with three different drugs.
Jurkat cells were separately incubated for 48 h with three different drugs, and the viable cells were counted manually using a haemocytometer; we performed this experiment three times. Cells (56%) were viable in 1 µM PKI402, 68% were viable in 1 µM everolimus, and 79% were viable in 1 µM PP242 (Figure 1).

The cell cycle experiment was repeated three times using Jurkat cells, which were incubated separately for 48 h with three different drugs to observe the cell cycle stage at which arrest occurred. Between 50% and 61% of the cells incubated with 1 µM of everolimus or 1 µM of PKI402 were arrested in G1 phase. On the other hand, 50% of the cells incubated with 1 µM PP242 were arrested in S phase (Figure 2).

3.2 The dual target inhibitor PKI402 was more effective at reducing the phosphorylation of Akt and S6 than single molecule inhibitors

![Percentage of positivity for pAkt and pS6 in Jurkat cell line](image)

**Figure 3:** Percentage of positivity for pAkt and pS6.

After determining the cell cycle differences between differently treated T-ALL Jurkat cells, we conducted phosphorylation experiments. We separately incubated the cells for 24 h with each of the three drugs, and measured the effects of these drugs using the two different antibodies, namely, α-pAkt and α-pS6. This experiment
tested the effectiveness of each drug at phosphorylation inhibition. For Jurkat cells incubated with 1 µM of everolimus, α-pAkt intensity was 64% and α-pS6 intensity was 37%, as measured by fluorescent label intensity. When the cells were treated with 1 µM of PKI402, the α-pAkt and α-pS6 fluorescence intensities were both ~30%. Cells treated with 1 µM PP242 exhibited 70% α-pAkt intensity and 50% α-pS6 intensity (Figure 3).

3.3 The IncRNA expression levels differed between Jurkat cells treated with dual and single molecule inhibitors

![LncRNA expression fold changes](image)

**Figure 4:** Normalized fold changes in selected IncRNA expression levels in the 96-well plate experiment.

An additional study characterised the effects of the three drugs on IncRNA expression levels. RNA was extracted from the Jurkat cell lines. A portion of this RNA was converted to cDNA using the standard protocol of the GoScript Reverse Transcription System (Promega). Next, the cDNA was quantified using qPCR, cyber
green dye, SYBR Green PCR Master Mix, and SYBR Green RT-PCR Reagents Kit (Life technology). The results are shown in Supplementary Figure 1.

In addition to the 96-well plate experiment, seven of the lncRNAs exhibiting high fold changes (Figure 4) were picked for a validation qPCR experiment, using primers designed for each RNA. Everolimus and PP242 were also used. These two drugs were used to test for lncRNA expression differences between a single molecule and dual molecule inhibitor.

3.4 The lncRNA expression levels differed between dual and single-molecule inhibitors

![qPCR results for different LncRNA](image)

**Figure 5:** qPCR validation experiment using primers designed for each RNA molecule; 5a, cells were treated with PKI402; 5b, with everolimus; and 5c, with PP242.
The validation experiment revealed that when PKI402 was used, RNCR3 and HULC levels were significantly elevated, whereas ANRIL and Zfhx2as were significantly lowered (Figure 5a). Cells treated with everolimus exhibited significantly increased RNCR3, UCA1, and HOXA3as levels (Figure 5b). The PP242 treated cells exhibited the greatest increases to RNCR3 and HULC levels of all treated cells, whereas ANRIL levels in these cells were significantly lowered (Figure 5c).

3.5 Zfhx2as IncRNA may be involved in increasing apoptosis and decreasing cell proliferation

![Graphs showing cell proliferation and apoptosis analysis](image)

**Figure 6:** Zfhx2as IncRNA knockdown. After knockdown, cell proliferation decreases while apoptosis increases.

Knockdown of Zfhx2as using siRNA in T-ALL cells was associated with a 30% decrease in cell proliferation (Figure 6a), and a 3-fold increase in cell apoptosis as indicated by annexin V staining (Figure 6b).
4. DISCUSSION

Recently approved by the U.S. Food and Drug Administration (FDA) [25], everolimus (RAD001) is a first-generation rapalog mTOR inhibitor with improved pharmacokinetics and reduced immunosuppression effects. This drug targets mTORC1 only [25]. The effect of everolimus on the Jurkat cell line was as expected. The fluorescence intensity indicating S6 phosphorylation was significantly lower (30%) than that indicating pAkt phosphorylation. These results are similar to those reported by Daver and colleagues [27]. One reason for these results would be the specificity of everolimus to mTORC1, which phosphorylates pS6 rather than pAkt; the latter is phosphorylated mainly by mTORC2 [25]. Cell proliferation, cell cycle progress, and expression of cyclins in G1 phase are regulated by PI3K/Akt/mTOR/p70S6K1 signalling pathways [28]. Everolimus was used to examine the effect of mTOR inhibitors on the T-ALL cell cycle, and the phase at which these cells arrest. The results obtained for proliferation and cell cycle experiments on Jurkat cells are similar to those reported by Huang and colleagues: everolimus inhibits T-ALL cell growth and arrests cells in G1 phase [29]. Furthermore, the effect of everolimus on the cell cycle is demonstrated by the 40% reduction in fluorescence intensity, which represents the amount of DNA in cells, in addition to the cause of G1 phase arrest.

PP242 is a second-generation mTOR protein kinase inhibitor; this class is selective for mTORC1/2 [25]. PP242 is a new small molecule targeting the mTOR ATP binding site, and specifically inhibiting the activities of both mTOR complexes. Activation of mTORC1 promotes cell proliferation through phosphorylation of S6K. mTORC2 plays an important role in cancer cell survival and proliferation through phosphorylation of Akt, which is required for maximal activation of the antiapoptotic kinase, enhancing cell survival and proliferation [30]. We find that PP242 reduces Jurkat cell viability by 20%, and inhibits proliferation.

We have also used PP242 to examine the effect of mTORC1/2 inhibitors on the T-ALL cell cycle, and the phase at which the cells arrest. We expected that most cells would arrest in the G1 phase because PI3K/Akt/mTOR/p70S6K1 signalling pathways regulate G1 phase expression cyclins. Unexpectedly, 50% of the cells arrested in the S phase. This result could be explained by the shortness of incubation time.
Furthermore, when PP242 inactivates mTORC1 and mTORC2, it also inhibits their direct (pS6K) and downstream (pAkt) targets \[^{[31]}\]. Fluorescent immunostains (Figure 3) indicate that PP242 inhibits one-half of pS6 phosphorylation and one-third of pAkt phosphorylation.

Phosphokinase inhibitor 402 is a second-generation mTOR inhibitor that works as a dual-specificity PI3K/mTOR inhibitor targeting PI3K, mTORC1, and mTORC2\[^{[26]}\]. Therefore, the dual-specificity inhibitors of mTOR and PI3K may be sufficient to evade the PI3K pathway reactivation by the mTOR-S6K1-IRS-1 negative feedback loop\[^{[25]}\]. The deregulation of PI3K activation has downstream effectors, including Akt and mTOR, which have been linked to tumour initiation and maintenance.

Phosphokinase inhibitor 402 is a small-molecule competitor with ATP in the PI3K/mTOR pathways, which suppresses phosphorylation of PI3K and mTOR effector proteins such as Akt. On the other hand, PKI402 can inhibit cell growth \[^{[31]}\]. Treating Jurkat cells with PKI402 demonstrated the dual specificity of PKI402 and emphasized the importance of PI3K/mTOR signalling in the cell cycle. In phosphorylation experiments, pS6 and pAkt antibodies were used to measure the phosphorylation activity after PKI402 treatment; both antibodies indicated that PKI402 inhibited phosphorylation by two-thirds. Phosphokinase inhibitor 402 was used to examine the effect of dual PI3K/mTOR inhibitors on the T-ALL cell cycle and determine at which stage the cells arrest. Half of the cells survived after incubation with PKI402. Half of these surviving cells were arrested in G1 phase. These results are explained by the mechanism of PI3K/Akt/mTOR/p70S6K1 signalling pathways that regulate cell proliferation, cell cycle progress, and cyclins expression in the G1 phase \[^{[28]}\].

The T-ALL cell line responds well to targeting therapies. These findings indicate that mTORC1 inhibitors, either alone or in combination with PI3K inhibitors, are potential therapeutics for T-ALL. However, dual-specificity inhibitors such as PKI402 also hold promise for treating T-ALL.

Recent genomic analysis found that only 1–2% of the human genome encodes protein-coding RNA, while the remaining transcriptome is non-protein-coding. Long non-coding RNAs (>200 bp long) are among the most abundant of the non-coding RNAs. Most IncRNAs are expressed in a tissue-specific and stress-specific manner.
Long noncoding RNAs regulate a wide range of biological functions; alterations to these functions can affect genomic imprinting and transcriptional regulation, potentially leading to cancer. Many lncRNAs are aberrantly expressed in a wide range of cancers. Some of these expressed lncRNAs are potential cancer biomarkers. To further understand the molecular interactions associated with these therapies, we screened 96 cancer-associated lncRNAs with PKI402 on Jurkat cell lines. Interestingly, we found that many cancer-associated lncRNAs are downregulated, and tumour suppressor lncRNA was either not changed or upregulated with treatment. RNCR3, UCA1, and HULC levels were increased by all three drugs. HOXA Cluster Antisense RNA (HOXAAS) is located between the HOXA cluster HOXA3 and HOXA4 genes. We found that everolimus regulated HOXAAS expression in leukaemic cells. HOXAAS reportedly regulates apoptosis during ATRA-induced myeloid differentiation. The dual inhibitor PKI402 significantly suppressed the expression of the zinc finger homeobox 2 antisense (Zfhx2as) lncRNA upon treatment. Zfhx2as is transcribed on the opposite strand and downstream of the Zfhx2 polyA site. Knockout of Zfhx2as reportedly induces Zfhx2 expression in mice. However, the role of Zfhx2as in cancer has not been evaluated until now. Interestingly, we found that knocking out Zfhx2as significantly decreases cell proliferation, and induces apoptosis in cancer cells. To the best of our knowledge, we are the first to report that Zfhx2as could act as a cancer-associated lncRNA with oncogenic properties.

We suggest future investigations on the effect of these drugs on induction and apoptosis. In addition, inhibitor studies on patient-derived cells would be informative. Combination studies of these inhibitors with selected conventional drugs may reveal interesting synergistic combinations.
5. REFERENCES


Supplementary Figure 1: Normalised fold changes for the lncRNA 96-well plate experiment (System bioscience).