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# Effects of imidazole derivatives on cellular proliferation and apoptosis in myeloid leukemia

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## Abstract

**Background** Acute promyelocytic leukemia (APL) is the sub-type of Acute myeloid leukemia (AML) which is described by differentiation block at promyelocytic stage and t(15; 17) translocation with All trans retinoic acid (ATRA) and arsenic trioxide (ATO) as standard treatments. Chronic myeloid leukemia (CML) translocation t (19; 22) causes a rise in granulocytes and their immature precursors in the blood. Different mutations cause resistance to first-line tyrosine kinase therapies in CML. Beside drug resistance, leukemia stem cells (LSC) are critical resources for relapse and resistance in APL and CML. The drug toxicity and resistant profile associated with LSC and current therapeutics of APL and CML necessitate the development of new therapies. Imidazoles are heterocyclic nitrogen compounds with diverse cellular actions. The purpose of this research was to assess the anti-leukemic properties of four novel imidazole derivatives including L-4, L-7, R-35, and R-NIM04.

**Methods and results** Pharmacological and biochemical approaches were used which showed that all four imidazole derivatives interfere with the NB4 cells proliferation, an APL cell line, while only L-7 exhibit anti-proliferative activity against K562 cells, a CML cell line. The anti-proliferative effect of imidazole derivatives was linked to apoptosis induction. Further real-time polymerase chain reaction (RT-PCR) analysis revealed downregulation of AXL-Receptor Tyrosine Kinase (AXL-RTK) and target genes of Wnt/beta-catenin pathway like *c-Myc*, *Axin2* and *EYA3*. An additive effect was observed after combinatorial treatment of L-7 with standard drugs ATRA or Imatinib on the proliferation of NB4 and K562 cells respectively which was related to further downregulation of target genes of Wnt/beta catenin pathway.

**Conclusion** Imidazole derivatives significantly reduce proliferation of NB4 and K562 cells by inducing apoptosis, down regulating of AXL-RTK and Wnt/ $\beta$ -catenin target genes.

**Keywords** Leukemia, Imidazole, Cancer therapy

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## Introduction

APL is characterised by t(15; 17) and differentiation block at promyelocytic stage. APL accounts for approximately 10–15% of all AML cases. The t(15; 17) translocation results in leukemia-associated fusion protein (LAF), PML-RAR $\alpha$  [1, 2]. So far, ATRA, ATO and chemotherapy are the treatment options for newly diagnosed APL cases [3]. Clinically, ATRA and ATO have been useful for treatment of patients with low and intermediate risk APL. Patients having high risk APL are treated with ATRA, ATO and chemotherapy [4]. ATO alone or combined with either ATRA or chemotherapy followed by stem cell transplant was taken in consideration for relapsed APL. ATO alone achieved complete remission (CR) in 80% of relapsed patients. However, 10–20% of patients with relapsed APL are not sensitive to ATRA and ATO either alone or in combination. Despite the availability of standard treatment options, problems such as differentiation syndrome, treatment resistance, relapse, and premature death persist in APL [5, 6].

CML is a myeloproliferative disease that increases granulocytes and their immature precursors in the blood. CML involves t(9;22)(q34;q11.2) translocation, known as Philadelphia chromosome (Ph) which is a constitutive active kinase and causes increased proliferation by activation of downstream signalling [7, 8]. Tyrosine kinase inhibitors (TKIs) are standard treatment options for chronic phase CML. Ponatinib, a third generation TKI targeting the T315I mutation, is used to treat CML if patients develop resistance to first-generation (Imatinib) and second-generation (nilotinib, dasatinib) inhibitors [9]. Furthermore, Imatinib and dasatinib are unable to target CML LSC, but ponatinib, a multikinase inhibitor can slightly interfere with CML LSCs [10]. Anyhow CML LSCs are still problem of relapse in CML.

Several studies reported that Wnt/ $\beta$ -catenin pathway play a pivotal role in cancer progression. It is well understood that activation of Wnt signaling leads to pathogenesis and maintain LSCs in AML and CML [11–13]. Since inhibition of Wnt signaling directly targets LSC, drugs and similar signaling pathways that can inhibit Wnt signaling offer significant potential within the setting of maintenance therapy for leukemia. Therefore, a small molecule that effectively inhibits Wnt/ $\beta$  catenin signaling might provide a desirable therapeutic option.

The side effects, drug toxicity, and resistance profiles associated with currently available anticancer

therapeutics for APL and CML necessitate the development of novel anticancer drugs. Imidazoles are nitrogen-containing heterocyclic rings that exhibit various therapeutic properties and have anticancer potential as well [14]. These interesting anticancer properties of imidazole inspired the development of imidazole derivatives with the goal of reducing side effects and increasing treatment efficacy. This study was designed to assess the anti-cancer potential of imidazole derivatives against APL and CML cell lines.

## Materials and methods

### Chemicals and cell lines

K562 cell line was used as a model for BCR/ABL1 positive CML and NB4 for PML/RAR $\alpha$  positive APL. The cell lines were obtained from our laboratory stock vials purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). RPMI 1640 media having 10% fetal bovine serum (FBS; Gibco/Invitrogen, Karlsruhe, Germany), 1% Penicillin/Streptomycin (P/S) and 1% L-glutamine was used to culture cell lines. Our collaborators provided imidazole derivatives, which were diluted in ethanol (Sigma, Steinheim, Germany). The stock solution was diluted to 1X assay concentrations. ATRA (Sigma Aldrich) was diluted in absolute ethanol according to manufacturers instructions. Imatinib obtained from Selleck Chemicals (Houston, TX, USA) was diluted in DMSO to 1000X stock which was further diluted to 1X working concentration for experiments.

### Trypan blue exclusion assay

Trypan blue exclusion assay were carried out before MTT assay, to assess cell viability as mentioned [15].

### MTT assay

The impact of imidazole derivatives on NB4 and K562 cells proliferation was checked through MTT cell proliferation assay kit (Roche, Basel, Switzerland). Approximately 10,000 cells were cultured in media containing 10% FBS 1% P/S, 1% L-glutamine and were plated in 96-well plates with or without inhibitors (alone or in combination) at the concentrations mentioned in Table 1. The cells were then incubated for 72 h. As a control, cells were plated in media containing 0.01% of solvent of the imidazole derivatives. After 72 h, cells were incubated with 15  $\mu$ l of filter-sterilised MTT (5 mg dissolved in 1ml PBS) inside a CO<sub>2</sub> incubator (Galaxy 170 R, New Brunswick) for 3–4 h at 37 °C. 150  $\mu$ l of DMSO was added to dissolve crystals after careful removal of MTT medium from all wells without disturbing the crystals. Before measuring the absorbance, the 96-well plate was incubated for 15 min. Using a micro-plate reader, absorbance was measured at reference wavelength of 550 nm and

**Table 1** Various ATRA, Imatinib and L-7 concentrations used in single targeting

Inhibitor	Concentrations ( $\mu$ M)		
ATRA	0.5	1	2
Imatinib	0.125	0.25	0.5
L-7, L-4, R-35, R-NIMO4	1.25	2.5	5

**Table 2** Key considerations for primer design in gene amplification

Gene / Primer	Primer Sequence	GC content	Annealing Temperature
$\beta$ -actin-FW	CATGTACGTTGCTATCC AGGC	52.38%	59.13 °C
$\beta$ -actin-RV	CTCCTTAATGTCACGCA CGAT	47.62%	58.46 °C
AXL-FW	GTCGGACCACTGAAGC TACC	60.0%	60.1 °C
AXL-RV	CATCGTCTTCACAGCC ACCT	55.0%	60 °C
EYA3-FW	GGGCAAGAGGAAAG CTGA	55.6%	59.9 °C
EYA3-RV	GCCACTGAAACCATCT GTTG	50%	60.4 °C
c-Myc-FW	CAGCGACTCTGAGGAG GAAC	60.0%	59.8 °C
c-Myc-RV	TCGGTTGTTGCTGATC TGTC	50.0%	58.2 °C
AXIN 2-FW	TCAAGTGCAAACTTTCG CCAACC	47.8%	62.8 °C
AXIN2-RV	TAGCCAGAACCTATGTG ATAAGG	43.4%	57 °C

660 nm (PR 4100 Bio-Rad). To analyze the data, the control/un-treated cells proliferation was used as a baseline (set to 1), while proliferation potential of the treated cells was shown relative to the baseline.

### Extraction of RNA for gene expression analysis

Two million cells were treated with imidazole derivatives and incubated till the doubling time of the respective cell lines; a control was also taken the same way. The cells were centrifuged for 5 min at 1500 rpm. For total RNA extraction, the supernatant was gently removed and pellet was resuspended in 1 ml of TRIzol reagent (Life Technologies) and RNA was extracted following standard protocol. At room temperature, the obtained RNA pellet was air-dried in a laminar flow hood. The pellet was dissolved in 20  $\mu$ l of NF water. Visual inspection of 2% agarose gel electrophoresis bands determined RNA quality. The extracted RNA was quantified through a Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, USA), and RNA purity was assessed by calculating the 260/280 ratio. RNA was then stored at -80 °C till future processing.

### Synthesis of cDNA

1  $\mu$ g of RNA template was used to synthesize complementary DNA (cDNA) using the WizBio cDNA Synthesis Kit, as per manufacturer's instructions and the quality of synthesized cDNA was verified. Primers were optimized for the housekeeping gene  $\beta$ -actin, as well as for *AXL-RTK*, *EYA3*, and *c-Myc*, using standard polymerase chain reaction (PCR) protocols.

### Analysis of gene expression

To explore the gene expression at the transcriptional level, a real-time polymerase chain reaction (RT-PCR) (qPCR; Applied Biosystems, Darmstadt, Germany 7300) was used. SYBR Green master mix (5X) was utilized for qRT-PCR (Solis BioDyne). The qRT-PCR program included an initial cycle of 50 °C for 2 min and 95 °C for 3 min followed by 40 cycles of amplification for 10 s at 95 °C and afterwards 60 °C for 1 min. Table 2 contain the primers sequences used to express the aforementioned genes in NB4 and K562 cells. The Ct values were exported to Microsoft Excel worksheet for analysis of the fold change according to comparative Ct method of calculating fold changes. The target genes expression was normalised to endogenous  $\beta$ -actin gene given by  $2^{-\Delta\Delta Ct}$ .

### Apoptosis analysis

To assess apoptosis, DNA fragmentation assay was performed on K562 and NB4 cells seeded in a 6-well plates and treated with inhibitors. After 72 h incubation, DNA isolation was carried out using the method described [16]. The pellet was air-dried approximately for 30 min, followed by resuspension in 40  $\mu$ l of NF water and then quantified through a Nanodrop 2000 (Thermo Fisher Scientific, USA). For gel electrophoresis, equal amounts of DNA were loaded into wells on a 1.5% agarose gel which was examined using UV transilluminator [16].

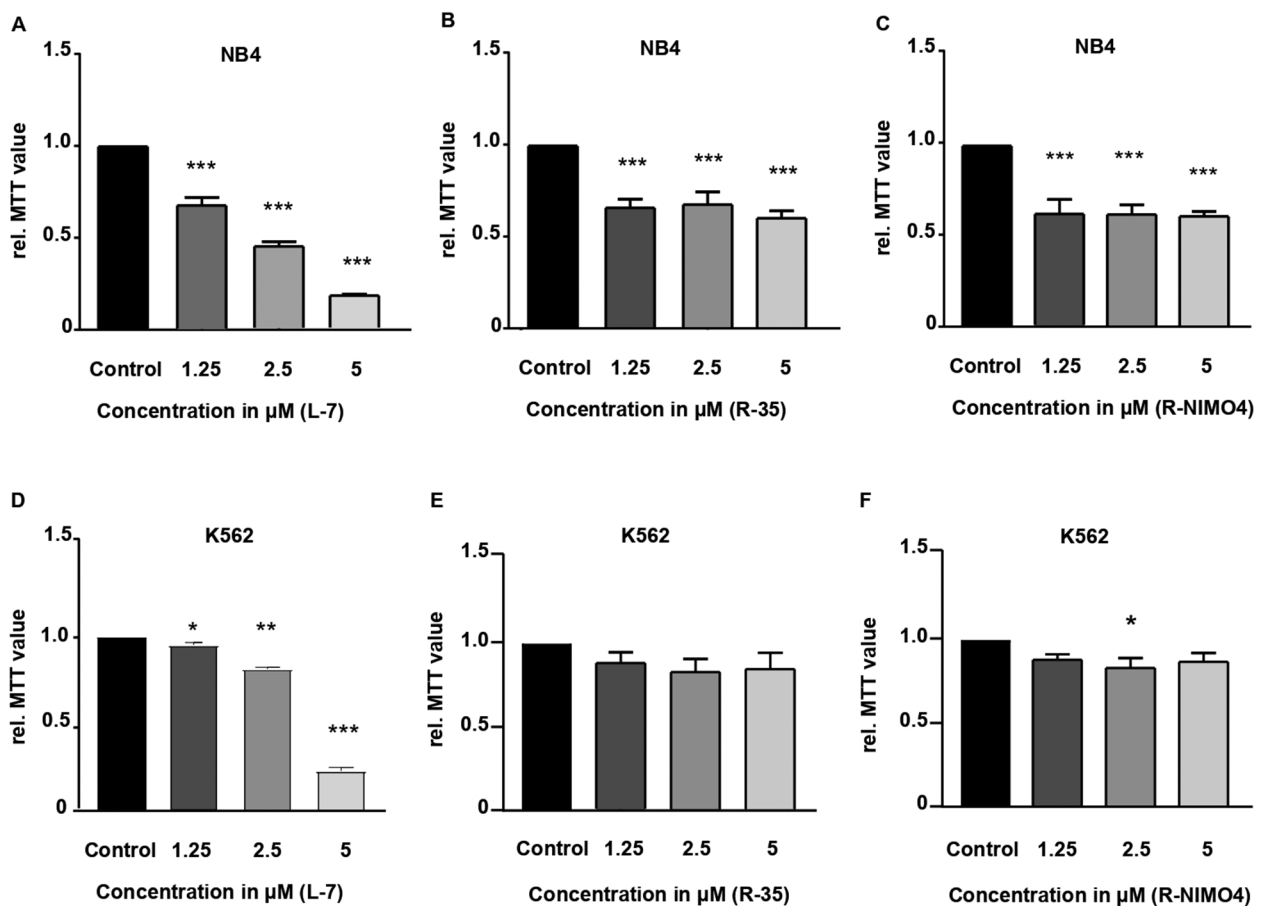
### Statistical analysis

Each experiment was carried out in triplicates, both technically and biologically. The findings were tabulated as mean  $\pm$  standard deviation. Data was analysed through One-way ANOVA and Student t-test using GraphPad Prism5 (GraphPad Software Inc. San Diego, CA) with p-value less than 0.05 considered as significant.

### Results

Apoptosis induction by imidazole derivatives leads to a notable reduction in the expansion of NB4 and K562 cell lines.

Imidazole has been shown to have anticancer potential [17–19], therefore present study was aimed to explore the antiproliferative effect of imidazole derivatives in NB4 and K562 cells. For this reason, cells were treated with increasing concentrations (0, 1.25, 2.5 and 5  $\mu$ M) of L4, L7, R-35 and R-NIMO4. Cell proliferation and cytotoxicity were evaluated using MTT assay. Either ethanol or DMSO served as a solvent control. We observed that imidazole derivative, L-7 causes a significant reduction in NB4 cell growth in a dose-dependent pattern (Fig. 1A) while R-35 and R-NIMO4 showed a slight effect on NB4 cells (Fig. 1B and C). Similarly, K562 cells also exhibited a significant reduction in proliferation by L-7 in dose dependent manner as shown in (Fig. 1D). R-35



**Fig. 1** Effect of imidazole derivatives on proliferation potential of NB4 and K562 cells through MTT assay. (A) L-7 effect on NB4 cells (B) R-35 effect on NB4 cells. (C) R-NIMO4 effect on NB4 cells. (D) L-7 effect on K562 cells. (E) R-35 effect on K562 cells. (F) R-NIMO4 effect on K562 cells. Cells were cultured in RPMI 1640 medium (10% FBS, 1% P/S1% L-glutamine), 0.01% ethanol, and the indicated concentrations of L7. One-Way ANOVA was used to test statistical significance (statistically highly significant p-values are < 0.001). Mean  $\pm$  SEM are represented by bars. \* p-value < 0.05, \*\* p-value < 0.01, \*\*\* p-value < 0.001

and R-NIMO4 have slightly reduced the proliferation of K562 cells (Fig. 1E and F). L4 showed a slight effect on NB4 cells (Sup Fig. 1A) and no effect on K562 cells (Sup Fig. 1B). To evaluate, whether the anti-proliferative potential of imidazole derivatives on NB4 and K562 cells is associated with apoptosis induction, these cells were exposed to imidazole derivatives for 72 h and DNA fragmentation assay was performed to evaluate apoptosis. L4 and L7 were observed to induce apoptosis in NB4 cells (Sup Fig. 2A) while L7 induced apoptosis in K562 cells (Sup Fig. 2B).

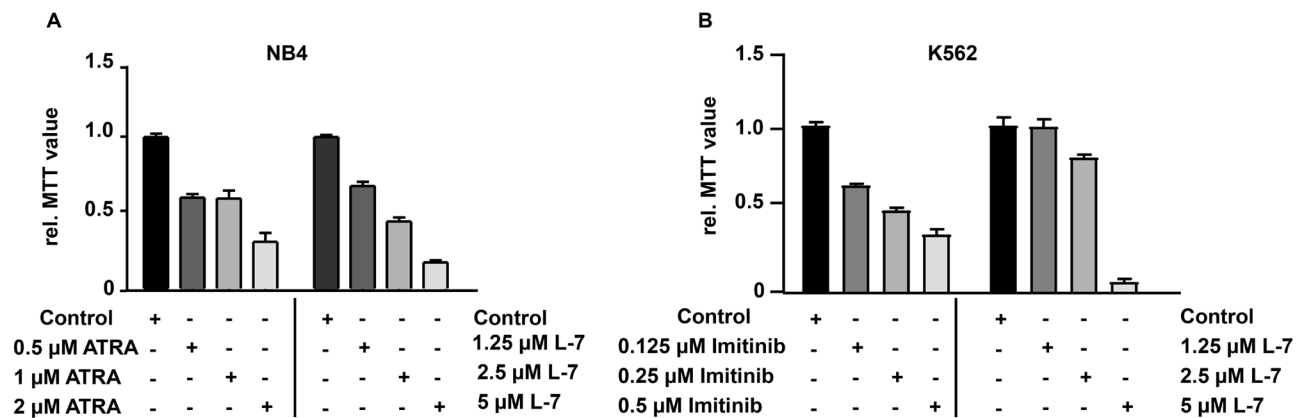
Overall, our results demonstrate that imidazole derivatives reduce the proliferation of NB4 and K562 cells by inducing apoptosis.

#### Comparison of the anti-proliferative activity of imidazole derivatives with ATRA or Imatinib in NB4 and K562 cells respectively

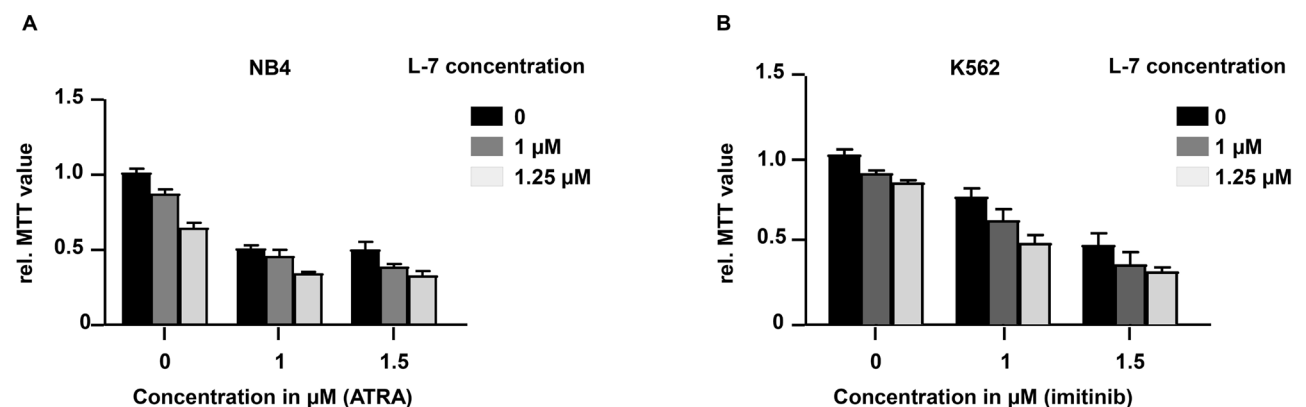
ATRA and Imatinib are standard drugs used in treatment of APL and CML respectively. We have shown that

imidazole derivative L-7 reduce proliferation of NB4 and K562 cells independently, therefore we compared the antiproliferative effects of imidazole derivatives with ATRA in NB4 and Imatinib in K562 cell lines. For this purpose, NB4 cells were treated with ATRA or L-7, L-4, R-35, R-NIMO4 and K562 cells with Imatinib or L-7 as shown in (Fig. 2 and Sup Fig. 3A, B and C), and MTT assay was performed. From Fig. 2A, the results indicate that L-7 showed strong effects as compared to ATRA while other L-4, R-35 and R-NIMO3 showed less effect as compared to ATRA in NB4 cells (Sup Fig. 3A, B and C). Similarly, L-7 showed stronger effect as compared to Imatinib in K562 cells.

Taken together our results showed that L-7 has stronger antiproliferative effect on NB4 and K562 cell lines as compared to ATRA and Imatinib.



**Fig. 2** Comparison of antiproliferative activity between L-7 and ATRA, L-7 and Imatinib. (A). Comparison between L7 and ATRA on NB4 cells (B). Comparison between L7 and imatinib on K562 cells. Cells were cultured in RPMI 1640 medium (10% FBS, 1% P/S, 1% L-glutamine), 0.01% ethanol, and the given concentrations of L7, ATRA and Imatinib. MTT assay was performed after 72 h to evaluate cell proliferation.



**Fig. 3** Single and combined treatments Effect on NB4 cells with L7 and ATRA (A) and K562 cells with L7 and imatinib (B). Cells were cultured in RPMI 1640 medium (10% FBS, 1% P/S, 1% L-glutamine), 0.01% Ethanol, and given concentrations of L7, ATRA and Imatinib. MTT assay was performed after 72 h to measure cell proliferation

### Combinatorial targeting of NB4 cells with L-7 and ATRA and K562 cells with L-7 and Imatinib show additive antiproliferative effect

Our findings demonstrate that L-7 significantly reduces the proliferation of NB4 and K562 cells. ATRA and Imatinib are approved drugs for APL and CML and interfere with proliferation of NB4 and K562 cells respectively. To minimize the side effects of ATRA and Imatinib, we were interested to study combinatorial effects of imidazole derivatives and ATRA on NB4 cells and imidazole derivatives and Imatinib on K562 cells. NB4 cells were treated with the given concentrations of ATRA or imidazole derivatives (alone or in combination) as shown in (Fig. 3A and Sup Table 1) and performed MTT assay. We found that both ATRA and L-7 reduced NB4 cells proliferation in a dose-dependent fashion whereas combined treatment showed an additive antiproliferative effect (Fig. 3A). However, L-4, R-35 and R-NIMO4 could not show any additive antiproliferative effect on NB4 cells

(Sup Fig. 4A, B and C). Similarly, Imatinib and L-7 both reduced the proliferation of K562 cell line in a dose-dependent manner while combined treatment further reduced the proliferation of K562 cells and showed additive antiproliferative effect (Fig. 3B).

In conclusion, our findings suggest that combination of ATRA and L-7 effectively inhibits NB4 cells, while combination of Imatinib and L-7 can effectively inhibit K562 cells proliferation.

### Imidazole derivatives interfere with Wnt/beta catenin signalling in NB4 and K562 cells

Wnt  $\beta$ -catenin oncogenic signaling is indispensable for PML/RAR alpha and BCR/ABL induced leukemogenesis. Recently we and others have shown that AXL-RTK regulate Wnt signaling in APL and CML [20] and our results suggest that imidazole derivatives interfere with leukemogenic potential of PML/RAR alpha and BCR/ABL1, therefore we were interested to explore AXL-RTK

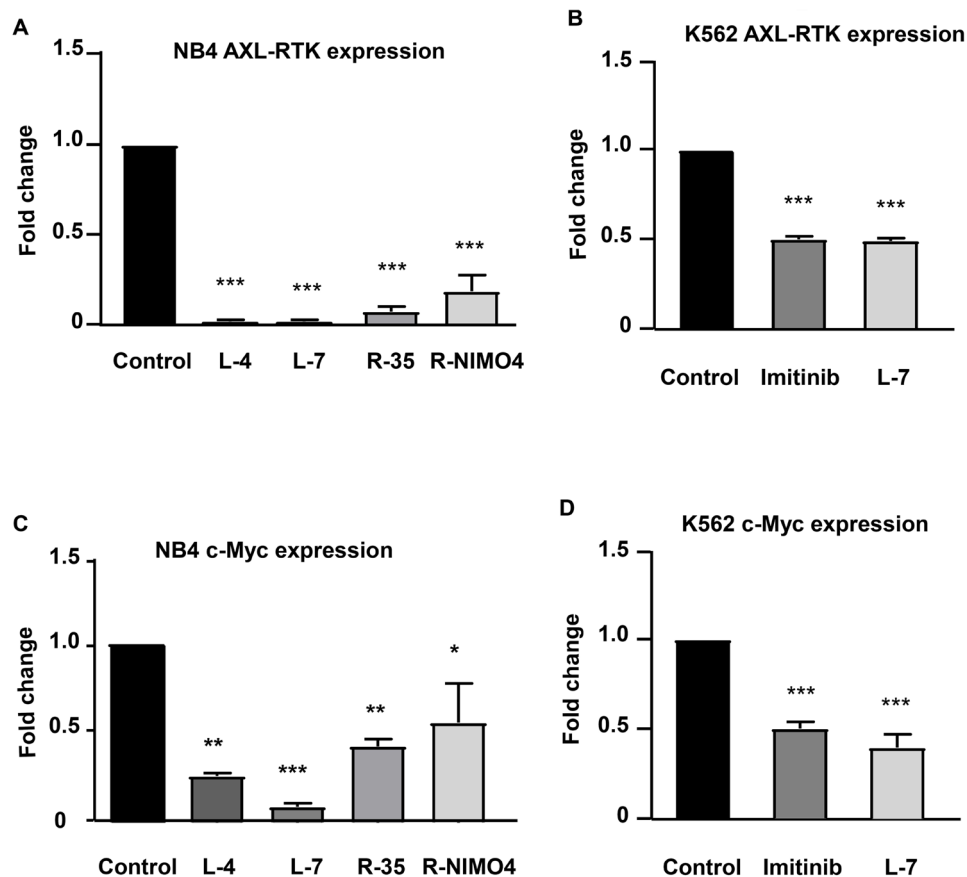
expression and Wnt signaling in APL and CML. Cells were plated and treated with given concentrations of imidazole derivatives. After 72 h RNA was extracted, and cDNA was synthesized. The Wnt target genes (*c-Myc* and *Axin2*) expression was assessed through RT-PCR. *AXL-RTK* was down regulated in NB4 and K562 cells as shown in Fig. 4A and B respectively, which in turn down regulated Wnt target genes (*c-Myc*) in NB4 and K562 cells (Fig. 5C, D), *Axin2* in K562 cells (Sup Fig. 6D). *EYA3* gene serves as a transcriptional regulator and co-activator of PP2A, which is a important cellular Ser/Thr phosphatase and involved in Wnt signalling by stabilizing *c-Myc*. Therefore *EYA3* gene expression in NB4 cells and K562 cells was investigated [18]. Expression of *EYA3* was assessed using RT-PCR. All imidazole derivatives reduced the expression of *EYA3* in NB4 cells except R-NIMO4 (Sup Fig. 5). Further single targeting of NB4 cells with L-7 was able to reduce significantly the expression of *c-Myc* and completely downregulated *EYA3* and combined targeting of NB4 cells with ATRA and L-7 showed the same results (Sup Fig. 6A and B). Similarly, treatment of K562 cells either with Imatinib or L-7

reduces the *c-Myc*, *aAxin2* and *EYA3* expression but co-targeting with Imatinib+L-7 results in further expression reduction of *c-Myc*, *Axin2* and *EYA3* (Sup Fig. 6C, D and E).

In summary we can conclude that L-7 interfere with Wnt/beta-catenin cellular signalling in NB4 and K562 cells by reducing the expression of *AXL-RTK*, *c-Myc*, *Axin 2* and *EYA3*.

## Discussion

The goal of current study was to evaluate the anti-cancer activity of four imidazole derivatives L7, L4, R-35, and R-NIMO4 against NB4 and K562 cell lines. Pharmacological and biochemical testing showed that all four compounds have anti-proliferative activity against NB4, while only L7 has the ability to reduce cellular proliferation in K562. The DNA fragmentation assay revealed that reduction in proliferation of NB4 and K562 cells is due to induction of apoptosis [19]. It was also observed that the combination treatments (L7+ATRA) and (L7+imatinib) had an additive effect on NB4 and K562 cell proliferation. Further, we could show that imidazole derivatives either



**Fig. 4** *AXL-RTK* and *c-Myc* Expression in NB4 and K562 cells after treatment with L4, L7, R-35, R-NIMO4 and Imatinib. Cells were cultured in RPMI 1640 medium (10% FBS, 1% P/S, 1% L-glutamine) 0.01% ethanol, and the indicated concentrations of L7, L4, R35 and R-NIMO4. Expression analysis was done through RT-PCR for NB4 cells (A and C) K562 cells (B and D). One way Anova was used to measure statistical significance. (statistically significant p-values are <0.05). Mean  $\pm$  SEM is represented as bars. \*\*\* p-value <0.001

alone or in combination with ATRA or imatinib reduce expression of *AXL-RTK* and interfere with Wnt signalling by down regulating *c-Myc*, *Axin2* and *EYA3* in NB4 and K562 cells.

Imidazoles are nitrogen-containing heterocyclic rings that are found in a variety of bioactive compounds and exhibit a variety of therapeutic properties including anti-cancer [13], antiviral [22], antibacterial [23], antiepileptic [24] and antifungal activities [25]. Several anti-cancer drugs, including fludarabine phosphate, dacarbazine, nilotinib, and ponatinib, have imidazole as a structural component [26]. Biochemical and pharmacological testing of imidazole derivatives show that all four compounds strongly interfere with the APL cell line (NB4) proliferation; while only L7 exhibit anti-proliferative activity against CML cell line (K562). The hallmarks of cancer include uncontrolled proliferation, angiogenesis and evasion of apoptosis. The loss of apoptotic control enables cancer cells to deregulate proliferation and interfere with differentiation [27]. Targeting and activating an apoptotic pathway in cancer cells could lead to a more universal treatment approach. Our findings suggest that imidazole derivatives induce apoptosis in NB4 and K562 cell lines which is in accordance with previous findings that different imidazole derivatives induce apoptosis in various cancers [28, 29].

*AXL-RTK* has been found to have a role in chemotherapy resistance in different cancers including ovarian cancer [30] and AML [31]. We have found that *AXL-RTK* is down regulated by imidazole derivatives and highlights importance of imidazole derivatives in resistant and refractory AML. Recent findings in breast cancer suggest that knockdown leads to a decrease in nuclear  $\beta$ -catenin whereas *AXL* stimulation by *Gas6* dramatically raised  $\beta$ -catenin levels and triggered its nuclear translocation demonstrating *AXL-RTK* involvement in both the development of cancer and the stabilization of  $\beta$ -catenin [20, 32–34]. Wnt/ $\beta$ -catenin signalling pathway is crucial in the maintenance of LSCs in the AML and CML. Our study revealed that imidazole derivatives downregulate *AXL-RTK*, which is related to downregulation of target genes of Wnt/ $\beta$ -catenin pathway, like *c-Myc* and *Axin2*. This suggests that *AXL-RTK* is involved in modulating the Wnt/ $\beta$ -catenin pathway in APL and CML. *EYA3* gene is member of a unique proteins family with dual functions like as co-activators for transcription and haloacid dehalogenase-like Tyr phosphatases. These proteins have different functions including cells proliferation and migration, invasion, and metastasis. The activity of *EYA3* as ser/thr phosphatase is not intrinsic but linked to PP2A [35]. *EYA3* stabilizes *c-Myc* by utilizing the phosphatase activity of PP2A, a major Wnt signalling regulator. Previous research has shown that  $\beta$ -catenin physically interacts with HIF-1 $\alpha$  at target gene sites and promotes

HIF-1 $\alpha$ -mediated transcription through a mechanism that is not fully understood [36]. Recent findings suggest that HIF-1 and 2 are required for the expression of *EYA3* (Yang C et al., 2022). Our findings suggest that imidazole derivatives down regulate HIF-1 $\alpha$  expression (data not shown), and except R-NIM04, all other imidazole derivatives/compounds showed significant decrease in *c-Myc*, *Axin2*, *EYA3* expression (Fig. 4, Sup Figs. 5 and 6) which may further confirm interference of imidazole derivatives with Wnt signalling. However, we showed that imidazole derivatives interfere with Wnt/ $\beta$  catenin pathway, the exact mechanism needs to be further explored. We investigated the role of imidazole derivatives in leukemic cell lines, further validation is required through primary samples of APL and CML patients and in in-vivo models. These imidazole derivatives have potential to contribute to the development of effective therapies for APL and CML in the future.

## Conclusion

Our findings indicate that imidazole derived compounds have the potential to decrease the proliferation ability of NB4 as well as K562 cells by down regulating *AXL-RTK* and by interfering with  $\beta$ -catenin dependent signalling in APL and CML.

## Abbreviations

APL	Acute promyelocytic leukemia
AML	Acute myeloid leukemia
CML	Chronic Myeloid Leukemia
AXL-RTK	Receptor tyrosine kinase
ATRA	All trans retinoic acid
ATO	Arsenic trioxide
LSC	Leukemia stem cells
RT-PCR	Real time polymerase chain reaction
LEAP	Leukemia-associated fusion proteins

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12885-024-12958-4>.

Supplementary Material 1  
Supplementary Material 2  
Supplementary Material 3  
Supplementary Material 4  
Supplementary Material 5  
Supplementary Material 6  
Supplementary Material 7

## Acknowledgements

None declared.

## Author contributions

Conception and design: D.K.; Development of methodology, B.B.N., A.B., M.K., G.R.S & D.K.; Acquisition of data B.B.N. & A.B; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.B.N., A.B., F.A., Z.A., D.K.; Writing Original Draft: B.B.N., A.B., M.K. & D.K.; Writing-Review and

Editing: Z.A., F.A., D.K.; Study supervision: D.K.; Performed experiments: B.B.N., A.B.; Others: B.B.N., A.B., M.K. Z.A., F.A., & D.K., Funding Acquisition: D.K. The author(s) read and approved the final manuscript.

#### Funding

The study was supported by a grant from Higher Education Commission of Pakistan (No: 21–1826/SRGP/R&D/HEC/2018) to Dilawar Khan Ph.D.

#### Data availability

All data generated or analysed during this study are included in this published article [and its supplementary information files].

#### Declarations

##### Ethics approval and consent to participate

Not applicable.

##### Consent for publication

Not applicable.

##### Competing interests

The authors declare no competing interests.

##### Protein sequences

Not applicable.

##### DNA and RNA sequences

Not applicable.

##### DNA and RNA sequencing data

Not applicable.

##### Genetic polymorphisms

Not applicable.

##### Linked genotype and phenotype data

Not applicable.

##### Macromolecular structure

Not applicable.

##### Microarray data (must be MIAME compliant)

Not applicable.

##### Crystallographic data for small molecules

Not applicable.

Received: 18 December 2023 / Accepted: 17 September 2024

Published online: 28 September 2024

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