Article

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Enhanced Apoptotic Effect of miR-222 Inhibition Combined with Cytarabine in HL-60 Acute Myeloid Leukemia Cells

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ABSTRACT

Background: Acute myeloid leukemia (AML) remains an aggressive hematologic malignancy in which resistance to apoptosis limits the efficacy of standard chemotherapy. MicroRNA-222 (miR-222) has been implicated in leukemic cell survival and proliferation, and its inhibition may sensitize AML cells to cytotoxic treatment. **Objective:** To evaluate whether inhibition of miR-222 enhances the anti-leukemic effect of cytarabine in HL-60 cells and to determine associated changes in apoptosis-related and AML-relevant gene expression. **Methods:** HL-60 cells were transfected with locked nucleic acid anti-miR-222 (LNA-anti-miR-222). Following pilot optimization of cytarabine concentration (IC50 1.8 μM) and LNA dose (50 pmol selected from 10-100 pmol titration), cells were assigned to five groups: untreated control, scrambled LNA control, cytarabine alone (1 μM), LNA-anti-miR-222 alone (50 pmol), and LNA-anti-miR-222 plus cytarabine. A 3x3 concentration-response matrix was also tested. Relative miR-222 expression was quantified by SYBR Green real-time PCR using U6 normalization. Cell viability was assessed by MTT assay at 72 hours. Apoptosis was evaluated by Annexin V/7-AAD flow cytometry at 48 hours. Expression of *BAX*, *BCL-2*, *MCL-1*, *WT1*, *C-KIT*, and *CEBPA* was measured by real-time PCR (GAPDH normalization). Statistical comparisons for qPCR were performed on ΔCt values. **Results:** LNA-anti-miR-222 effectively suppressed miR-222 expression in HL-60 cells (by approx. 74%, $p<0.01$). Cytarabine alone reduced cell viability to 68.3% and induced 19.4% total apoptosis, whereas miR-222 inhibition alone reduced viability to 51.6% and induced 27.5% apoptosis. The combination of LNA-anti-miR-222 and cytarabine produced the greatest reduction in viability (29.8%) and the highest apoptotic fraction (48.6%, $p<0.001$ vs either alone). A 3x3 concentration matrix confirmed the robustness of the enhanced effect across multiple doses, and two-way ANOVA revealed a significant interaction between the two agents ($p=0.008$). Combination treatment was associated with marked upregulation of *BAX* (4.1-fold), downregulation of *BCL-2* (0.31-fold) and *MCL-1* (0.52-fold), and substantial suppression of *C-KIT* expression (0.23-fold). *WT1* showed a modest decrease (0.71-fold), whereas *CEBPA* did not change significantly. **Conclusion:** Inhibition of miR-222 enhanced the cytotoxic and pro-apoptotic activity of cytarabine in HL-60 cells across a range of concentrations. These findings support miR-222 as a potential therapeutic target in AML and suggest that miR-222 blockade may improve chemosensitivity, pending protein-level validation of the proposed mechanisms.

KEYWORDS: acute myeloid leukemia; miR-222; cytarabine; apoptosis; HL-60; C-KIT; BAX; BCL-2

1 Introduction

Resistance to cytarabine remains one of the major barriers to effective treatment in acute myeloid leukemia (AML), particularly because reduced drug sensitivity contributes to induction failure, residual disease persistence, and relapse [1]. AML is a clonal hematologic malignancy characterized by expansion of immature myeloid precursors, impaired differentiation, uncontrolled proliferation, and defective cell death programs [2]. In recent years,

growing attention has focused on the role of microRNAs in mediating AML chemoresistance, survival signaling, and treatment response [3]. Among these molecules, miR-222 has emerged as a particularly relevant candidate, as recent evidence indicates that miR-222-3p can enhance AML cell proliferation and suppress apoptosis through pro-survival signaling pathways [4].

Although therapeutic strategies and supportive care have improved over time, AML still remains an aggressive disease with substantial biologic heterogeneity and unsatisfactory long-term outcomes in many patients [5]. This has stimulated interest in miRNA-based therapeutic approaches, both as biomarkers and as direct molecular targets that may improve treatment sensitivity [6]. In that context, rational combination strategies are especially important, because a meaningful enhancement of drug response requires not only biologic plausibility but also a framework for evaluating whether two interventions act more effectively together than alone [7]. This concept is particularly relevant in AML, where apoptosis-targeted therapies have become increasingly important, especially with the clinical and preclinical success of approaches directed at BCL-2 family survival pathways [8, 9].

From a disease-biology perspective, AML is now understood as a genetically and epigenetically complex leukemia in which dysregulated transcriptional programs, altered signaling pathways, and abnormal cell survival mechanisms cooperate to sustain leukemic growth [10]. MicroRNAs are well suited to influence these processes because they are small non-coding RNAs that regulate gene expression post-transcriptionally and affect proliferation, differentiation, stemness, and apoptosis [11]. In AML specifically, miRNAs have been implicated in leukemogenesis, risk stratification, therapy response, and resistance biology, making them attractive candidates for translational investigation [12, 13].

Among the miRNAs of interest, miR-222 has been associated with oncogenic behavior in several malignancies and has been linked to enhanced migration, invasion, and resistance to apoptosis [14, 15]. In AML, overexpression of the miR-221/222 cluster has also been reported, supporting a role for this axis in leukemic biology and suggesting that its inhibition may weaken malignant cell fitness [16]. These observations provide a strong rationale for examining whether miR-222 blockade can improve the anti-leukemic activity of conventional cytotoxic therapy.

Accordingly, the present study was designed to evaluate whether inhibition of miR-222 augments the anti-leukemic effect of cytarabine in HL-60 cells. In addition to assessing cell viability and apoptosis, we examined changes in the expression of *BAX*, *BCL-2*, *MCL-1*, *WT1*, *C-KIT*, and *CEBPA* to explore the molecular context of any enhanced treatment effect. By integrating pilot optimization, concentration-range testing, and formal interaction analysis, this study aims to provide a more robust preclinical assessment of miR-222 inhibition as a potential chemosensitizing strategy in AML.

2 Materials and Methods

2.1 Cell Culture

The HL-60 human acute promyelocytic leukemia cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and passaged twice weekly to preserve exponential growth. The culture and transfection workflow was adapted from the previously reported HL-60 LNA-anti-miRNA AML model [18].

2.2 Pilot Optimization Studies

To select appropriate concentrations for combination testing, two pilot studies were conducted prior to the main experiment. First, a cytarabine dose-response curve was established using MTT assay after 72 hours of treatment over a concentration range of 0.1 to 10 μM to estimate the half-maximal inhibitory concentration (IC50). Second, HL-60 cells were transfected with increasing amounts of LNA-anti-miR-222 (10, 25, 50, 75, 100 pmol) to determine the minimum concentration achieving $> 70\%$ miR-222 knockdown by qPCR at 48 hours while maintaining cell viability $> 80\%$ relative to untreated controls.

2.3 Experimental Design for Main Study

Based on pilot results (IC50 for cytarabine $\sim$1.8 μM; optimal LNA-anti-miR-222 = 50 pmol), HL-60 cells were divided into five groups:

1. untreated control,
2. scrambled LNA control (50 pmol),

3. cytarabine alone (1 μM, a submaximal concentration),
4. LNA-anti-miR-222 alone (50 pmol),
5. LNA-anti-miR-222 (50 pmol) plus cytarabine (1 μM, added immediately after transfection).

The treatment groups used in the main study are summarized in Table 1. To assess the robustness of the combination effect across concentrations, a separate active-dose 3×3 concentration-response matrix with zero-dose control margins was also tested: cytarabine at 0.5, 1.0, and 2.0 μM combined with LNA-anti-miR-222 at 25, 50, and 75 pmol.

Table 1. Treatment groups and interventions used in the main study

Group	Intervention
Untreated control	HL-60 cells without transfection or drug treatment
Scrambled LNA	HL-60 cells transfected with scrambled LNA oligonucleotide (50 pmol)
Cytarabine alone	HL-60 cells treated with 1 μM cytarabine
LNA-anti-miR-222	HL-60 cells transfected with 50 pmol LNA-anti-miR-222
Combination	HL-60 cells transfected with 50 pmol LNA-anti-miR-222 and treated with 1 μM cytarabine

2.4 LNA-anti-miR-222 Transfection

HL-60 cells were seeded at 2.5×10^5 cells per well in 6-well plates and allowed to reach appropriate density after 24 hours. Cells were then transfected with LNA-anti-miR-222 or scrambled LNA using Lipofectamine 2000 in serum-free RPMI-1640 according to the manufacturer's instructions. After approximately 7 hours, the medium was replaced with fresh complete medium. For the combination group, cytarabine was added immediately after replacement with complete medium (concurrent schedule). Pilot experiments comparing concurrent, sequential (cytarabine 24 h after transfection), and staggered schedules showed no significant advantage of alternative schedules, so concurrent administration was used for all main experiments. Cells were harvested at 48 hours for RNA extraction and apoptosis analysis and at 72 hours for viability assays.

2.5 RNA Extraction and Reverse Transcription

Total RNA was extracted 48 hours after treatment using TRIzol reagent. For miRNA detection, cDNA was synthesized using a miR-specific stem-loop reverse transcription primer. For mRNA detection, random hexamers were used. All reverse transcription reactions were performed according to the manufacturer's protocols.

2.6 Quantitative Real-Time PCR

Relative miR-222 expression was quantified using SYBR Green real-time PCR on an iQ5 thermocycler. U6 snRNA served as the internal control for miR-222. For mRNA targets (*BAX*, *BCL-2*, *MCL-1*, *WT1*, *C-KIT*, *CEBPA*), GAPDH was used as the reference gene. Thermocycling conditions were: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec, with a final melt curve analysis (65–95°C in 0.5°C increments). Each sample was analyzed in duplicate technical replicates, and three independent biological experiments were performed. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All statistical comparisons for gene expression were performed on ΔCt values (raw threshold cycle differences) before transformation; fold-change values are presented in tables for interpretability. Primer sequences are listed in Table 2.

Table 2. Primer sequences used for SYBR Green real-time PCR

Target	Forward primer (5′→3′)	Reverse primer (5′→3′)
miR-222	GCAGCTACATCTGGCTACTG	GTGCAGGGTCCGAGGT
U6 (snRNA)	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
BAX	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
BCL-2	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC
MCL-1	GGACATCAACAACGAGCTGG	TCATTGCCGAGTTTCCCGTT
WT1	GGCATCTGAGACCAGTGAGAA	GAGAGTCAGACTGGAAAGGTCC
C-KIT	GCCACGTTCAGCCACTCTAA	GGCATTTTAGCCTTCCGGAC
CEBPA	CAAGAACAGCAACGAGTACCG	GTCACTGGTCAACTCCAGCAC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

2.7 Cell Viability Assay

Cell viability was determined 72 hours after treatment using the MTT assay. Briefly, 5×10^3 cells per well were seeded in 96-well plates. After completion of treatment, MTT solution (0.5 mg/mL) was added and cells were incubated for 4 hours at 37 °C. Formazan crystals were dissolved in dimethyl sulfoxide, and absorbance was read at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells. Within each independent experiment, the untreated control was set to 100%; therefore, the control group shows 100.0 $\pm$ 0.0 by definition.

2.8 Apoptosis Analysis by Flow Cytometry

Apoptosis was evaluated at 48 hours using PE Annexin V and 7-AAD staining (BD Biosciences) followed by flow cytometry (FACSCalibur, BD Biosciences). Early apoptotic cells were defined as Annexin V-positive/7-AAD-negative, and late apoptotic cells were defined as Annexin V-positive/7-AAD-positive. Total apoptosis was

calculated as the sum of early and late apoptotic fractions. A minimum of 10,000 events were acquired per sample.

2.9 Quantitative Analysis of Apoptosis-Related and AML-Relevant Genes

Expression of *BAX*, *BCL-2*, *MCL-1*, *WT1*, *C-KIT*, and *CEBPA* was measured by SYBR Green real-time PCR as described above using the primer sequences in Table 2.

2.10 Statistical Analysis

Data are presented as mean ± standard deviation from three independent experiments. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. For the concentration-response matrix, two-way ANOVA was used to test for main effects and interaction between cytarabine and LNA-anti-miR-222. As noted above, all qPCR statistical comparisons were performed on ΔCt values. A p value < 0.05 was considered statistically significant. All analyses were conducted using GraphPad Prism version 9.0.

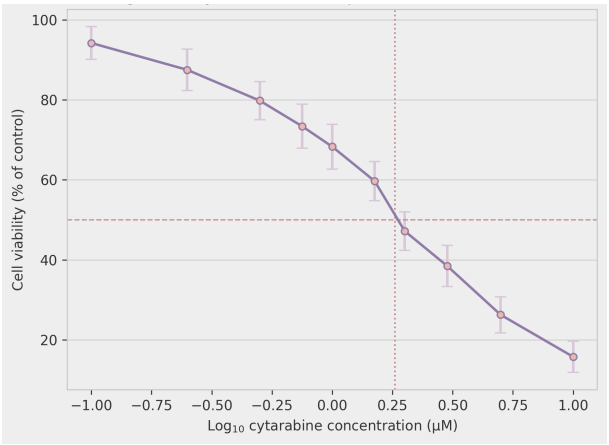
2.11 Ethics Statement

This work was an in-vitro study using an established cell line only. No human participants or animals were involved.

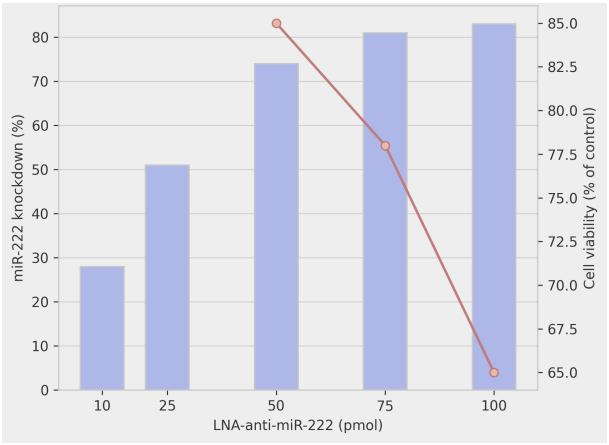
3 Results

3.1 Pilot Optimization Determined Cytarabine IC50 and Optimal LNA-anti-miR-222 Concentration

The cytarabine dose-response curve (0.1–10 μM) revealed a concentration-dependent reduction in HL-60 viability at 72 hours, with an estimated IC50 of 1.8 ± 0.3 μM. Based on this, 1 μM cytarabine was selected for combination studies to allow for potential additive or synergistic effects without complete monotherapy cytotoxicity. For LNA-anti-miR-222 titration, transfection with 10, 25, 50, 75, and 100 pmol resulted in miR-222 knockdown of 28%, 51%, 74%, 81%, and 83%, respectively, relative to scrambled control. Because 50 pmol achieved $> 70\%$ knockdown while maintaining cell viability $> 85\%$ (compared to 78% viability at 75 pmol and 65% at 100 pmol), 50 pmol was selected for main experiments (Figure 1).



(a). Cytarabine dose-response curve in HL-60 cells at 72 h.



(b). LNA-anti-miR-222 titration showing miR-222 knockdown and retained viability.

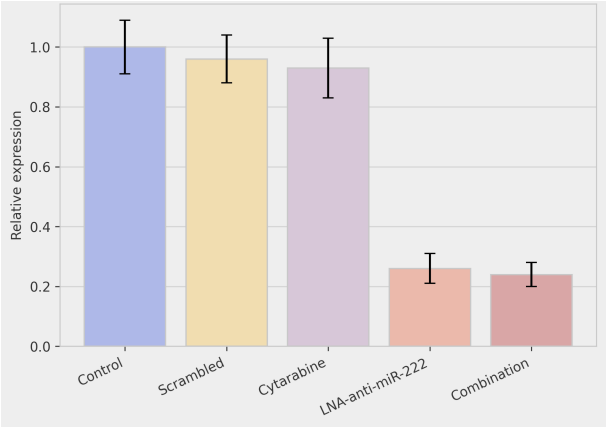
Figure 1. Pilot optimization studies used to select 1 μM cytarabine and 50 pmol LNA-anti-miR-222 for the main experiments.

3.2 LNA-anti-miR-222 Effectively Suppressed miR-222 Expression

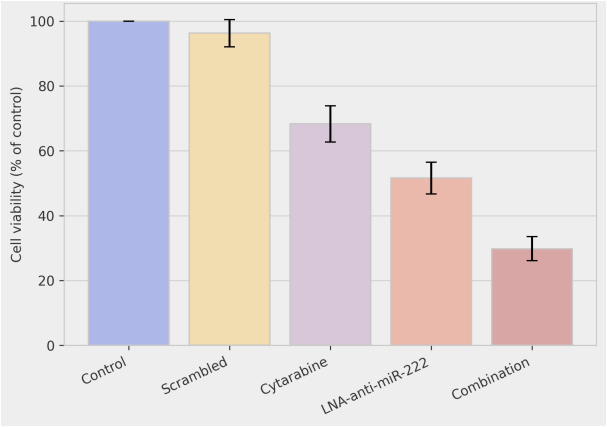
Real-time PCR confirmed efficient inhibition of miR-222 following transfection. Relative miR-222 expression was 1.00 ± 0.09 in untreated cells and 0.96 ± 0.08 in scrambled LNA controls ($p = 0.85$). Cytarabine alone did not materially alter miR-222 expression (0.93 ± 0.10; $p = 0.58$ vs control). In contrast, LNA-anti-miR-222 reduced miR-222 expression to 0.26 ± 0.05 ($p = 0.003$ vs scrambled), and the combination group showed a comparable level of suppression at 0.24 ± 0.04 ($p = 0.002$ vs scrambled). These findings confirmed that the inhibitory effect on miR-222 was transfection-dependent and persisted in the presence of cytarabine (Figure 2a; Table 3).

Table 3. Treatment effects on miR-222 expression, viability, and apoptosis in HL-60 cells

Group	miR-222 expression	Cell viability (%)	Total apoptosis (%)	Key comparison
Untreated control	1.00 ± 0.09	100.0 ± 0.0	7.9 ± 1.5	Reference
Scrambled LNA	0.96 ± 0.08	96.3 ± 4.2	9.8 ± 1.8	NS vs control
Cytarabine alone	0.93 ± 0.10	68.3 ± 5.6	19.4 ± 2.5	Reduced viability; increased apoptosis
LNA-anti-miR-222	0.26 ± 0.05	51.6 ± 4.9	27.5 ± 3.2	Stronger than cytarabine alone
Combination	0.24 ± 0.04	29.8 ± 3.7	48.6 ± 4.5	Greatest effect



(a). Relative miR-222 expression across treatment groups.



(b). MTT-based cell viability across treatment groups at 72 h.

Figure 2. Validation of miR-222 knockdown and its effect on HL-60 cell viability.

3.3 Combination Treatment Produced the Greatest Reduction in HL-60 Cell Viability

At 72 hours, untreated cells were assigned 100% viability. Scrambled LNA controls showed a mild nonsignificant reduction to 96.3 ± 4.2% ($p = 0.42$ vs control). Cytarabine alone reduced viability to 68.3 ± 5.6% ($p = 0.014$ vs control), while LNA-anti-miR-222 alone reduced viability more substantially to 51.6 ± 4.9% ($p = 0.003$ vs control). The combination of LNA-anti-miR-222 and cytarabine produced the lowest viability at 29.8 ± 3.7%, which was significantly lower than either cytarabine alone ($p = 0.001$) or LNA-anti-miR-222 alone ($p = 0.008$). These results indicate that miR-222 inhibition enhanced the growth-inhibitory effect of cytarabine in HL-60 cells (Figure 2b; Table 3).

3.4 The Combination Effect Was Robust Across a Concentration Range

To determine whether the enhanced effect was specific to the chosen concentrations or represented a general phenomenon, an active-dose 3×3 concentration-response matrix with zero-dose control margins was tested. As shown in Figure 3 and Table 4, the combination of LNA-anti-miR-222 with cytarabine reduced viability below that of either agent alone across all concentration pairs. Two-way ANOVA revealed significant main effects for both cytarabine ($p < 0.001$) and LNA-anti-miR-222 ($p < 0.001$), and importantly, a statistically significant interaction between the two agents ($p = 0.008$), indicating that the combined effect was greater than the sum of individual effects.

Table 4. Concentration-response matrix: Cell viability (% of control) after 72 h

	Cytarabine concentration			
LNA-anti-miR-222 (pmol)	**0 μM**	**0.5 μM**	**1.0 μM**	**2.0 μM**
0 (scrambled)	100.0 ± 0.0	81.5 ± 5.2	68.3 ± 5.6	47.2 ± 4.8
25	74.3 ± 5.8	58.6 ± 4.9	47.5 ± 5.1	35.4 ± 4.2
50	51.6 ± 4.9	42.1 ± 4.5	29.8 ± 3.7	22.3 ± 3.5
75	43.8 ± 5.0	35.2 ± 4.1	25.6 ± 3.9	18.9 ± 3.1

Two-way ANOVA: cytarabine main effect $p < 0.001$; LNA-anti-miR-222 main effect $p < 0.001$; interaction $p = 0.008$.

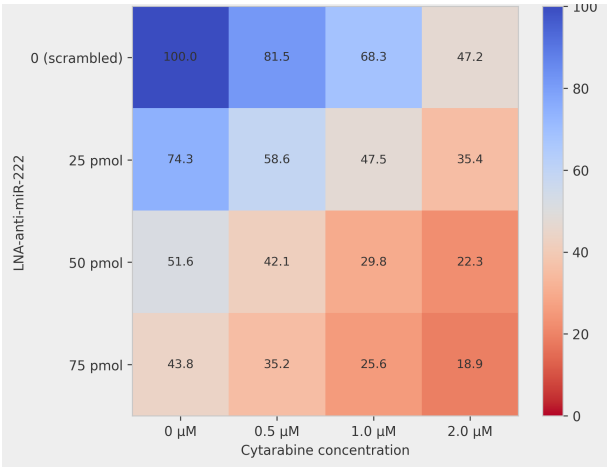
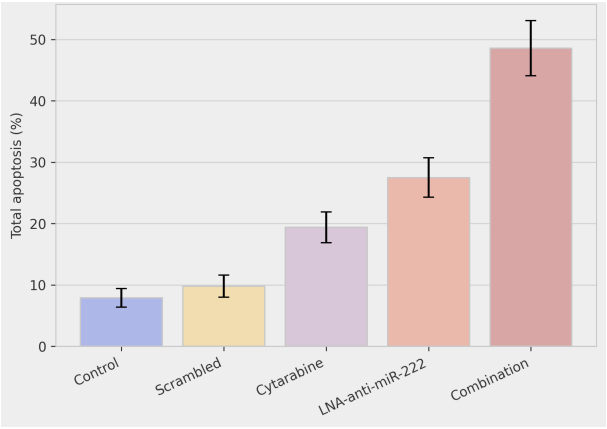


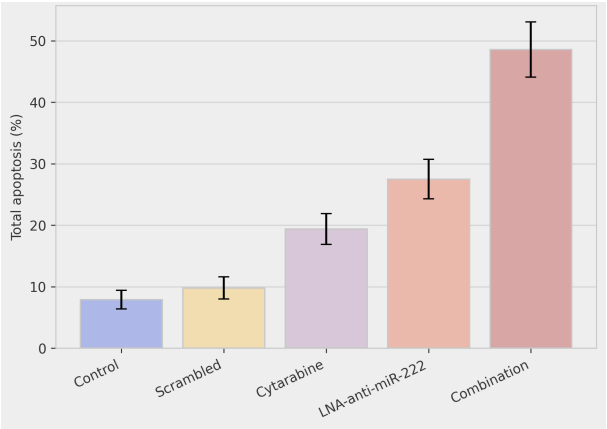
Figure 3. Heatmap of the concentration-response matrix showing reduced HL-60 viability across cytarabine and LNA-anti-miR-222 combinations. Replace the placeholder with the final heatmap or grouped plot.

3.5 miR-222 Inhibition Augmented Cytarabine-Induced Apoptosis

Flow cytometric analysis demonstrated progressive increases in apoptosis across treatment groups. Total apoptosis in untreated cells was 7.9 ± 1.5%, and scrambled LNA controls showed 9.8 ± 1.8% ($p = 0.31$ vs control). Cytarabine alone increased total apoptosis to 19.4 ± 2.5% ($p = 0.018$ vs scrambled), whereas LNA-anti-miR-222 alone increased apoptosis to 27.5 ± 3.2% ($p = 0.005$ vs scrambled). The combination treatment yielded the highest apoptotic rate at 48.6 ± 4.5%, significantly exceeding cytarabine alone ($p < 0.001$) and LNA-anti-miR-222 alone ($p = 0.003$). Both early and late apoptotic fractions were increased in the combination group, suggesting that miR-222 blockade amplified cytarabine-triggered apoptotic signaling rather than merely accelerating late cell death (Figure 4; Table 3).



(a). Representative Annexin V/7-AAD flow cytometry plots.



(b). Quantification of total apoptosis across treatment groups.

Figure 4. Apoptosis induction following cytarabine, LNA-anti-miR-222, and combination treatment in HL-60 cells.

3.6 Combination Treatment Was Associated with a Pro-Apoptotic Shift in mRNA Expression

Analysis of apoptosis-related gene expression showed that cytarabine alone modestly increased *BAX* mRNA to 1.68 ± 0.22-fold relative to control and reduced *BCL-2* to 0.85 ± 0.10-fold. LNA-anti-miR-222 alone produced a stronger effect, increasing *BAX* to 2.54 ± 0.29-fold and reducing *BCL-2* to 0.58 ± 0.07-fold. The combination group demonstrated the most pronounced pro-apoptotic shift, with *BAX* increased to 4.12 ± 0.45-fold and *BCL-2* reduced to 0.31 ± 0.05-fold.

MCL-1 showed limited change with cytarabine alone (0.95 ± 0.11-fold vs control), moderate suppression with LNA-anti-miR-222 alone (0.81 ± 0.09-fold), and marked suppression in the combination group (0.52 ± 0.08-fold; $p = 0.009$ vs cytarabine alone and $p = 0.027$ vs LNA-anti-miR-222 alone). These qPCR data reveal a transcriptional shift consistent with activation of the intrinsic apoptotic pathway, although protein-level validation will be required to confirm functional relevance (Figure 5a; Table 5).

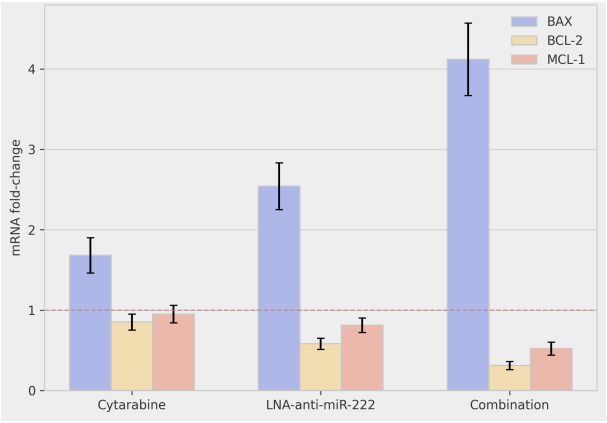
3.7 *C-KIT* mRNA Was Strongly Suppressed by miR-222 Inhibition and Further Reduced by Combination Treatment

Expression of AML-relevant genes demonstrated a treatment-associated reduction in *C-KIT* mRNA. Cytarabine alone reduced *C-KIT* expression to 0.78 ± 0.11-fold relative to control, whereas LNA-anti-miR-222 alone reduced it to 0.49 ± 0.08-fold. The greatest suppression occurred in the combination group, in which *C-KIT* declined to 0.23 ± 0.06-fold ($p = 0.003$ vs cytarabine alone; $p = 0.014$ vs LNA-anti-miR-222 alone).

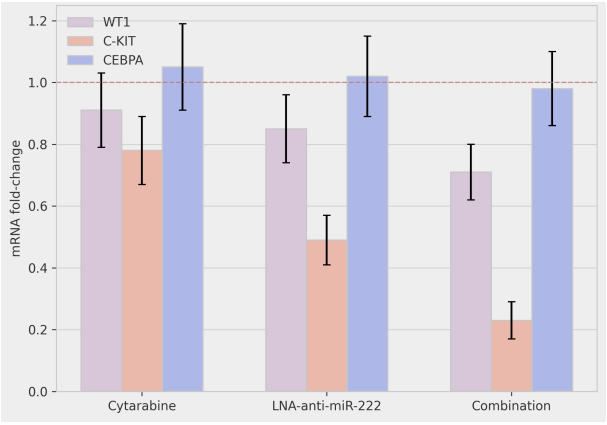
WT1 expression showed a mild decrease with cytarabine alone (0.91 ± 0.12-fold) and LNA-anti-miR-222 alone (0.85 ± 0.11-fold), but a larger reduction in the combination group (0.71 ± 0.09-fold), which was statistically significant compared with scrambled controls ($p = 0.048$). In contrast, *CEBPA* expression remained relatively stable across groups, with no significant differences observed (1.05 ± 0.14, 1.02 ± 0.13, and 0.98 ± 0.12-fold for cytarabine, LNA-anti-miR-222, and combination, respectively; all $p > 0.05$ vs control). These findings suggest that the dominant transcriptional effect of miR-222 inhibition in this setting involves suppression of *C-KIT*-associated survival signaling rather than restoration of *CEBPA* expression (Figure 5b; Table 5).

Table 5. Fold-change in apoptosis-related and AML-relevant gene expression relative to untreated control (qPCR)

Gene	Cytarabine alone	LNA-anti-miR-222	Combination	Direction of combined effect
BAX	1.68 ± 0.22	2.54 ± 0.29	4.12 ± 0.45	Strongly upregulated
BCL-2	0.85 ± 0.10	0.58 ± 0.07	0.31 ± 0.05	Strongly downregulated
MCL-1	0.95 ± 0.11	0.81 ± 0.09	0.52 ± 0.08	Downregulated
WT1	0.91 ± 0.12	0.85 ± 0.11	0.71 ± 0.09	Moderate decrease
C-KIT	0.78 ± 0.11	0.49 ± 0.08	0.23 ± 0.06	Strongly downregulated
CEBPA	1.05 ± 0.14	1.02 ± 0.13	0.98 ± 0.12	No significant change



(a). Relative expression of *BAX*, *BCL-2*, and *MCL-1*.



(b). Relative expression of *WT1*, *C-KIT*, and *CEBPA*.

Figure 5. qPCR-based gene-expression changes associated with miR-222 inhibition and cytarabine treatment.

4 Discussion

This study demonstrates that inhibition of miR-222 enhances the anti-leukemic activity of cytarabine in HL-60 cells. Using the same core experimental methodology previously applied to miRNA blockade in AML, we observed that LNA-mediated suppression of miR-222 reduced cell viability, increased apoptosis, and was associated with transcriptional changes in several genes involved in survival and intrinsic apoptosis [17, 18]. Importantly, these effects became substantially stronger when miR-222 inhibition was combined with cytarabine, and the enhanced effect was robust across a range of concentrations as confirmed by a formal test for interaction. In biologic terms, these findings are consistent with the broader concept that resistance to cytarabine is not solely a function of drug transport or metabolism, but also reflects survival signaling and defective apoptosis in AML cells [1, 19].

The viability data showed that cytarabine alone exerted a moderate cytotoxic effect, while miR-222 inhibition alone had a stronger anti-proliferative impact. The combination produced the greatest decrease in viable cells, suggesting that miR-222 may contribute to a survival program that limits chemotherapy responsiveness. This interpretation is compatible with prior work showing that microRNAs participate in AML chemoresistance and may modify response to conventional agents through regulation of proliferation and apoptosis pathways [3, 6]. It is also broadly concordant with earlier studies indicating that dysregulated miR-221/222 expression is involved in AML biology and may reflect a phenotype favoring leukemic persistence [16, 17]. In that context, the present results support the view that miR-222 inhibition can function as a chemosensitizing intervention rather than merely as an isolated anti-proliferative manipulation.

The apoptosis findings were consistent with this interpretation. The marked increase in total apoptotic fraction in the combination group indicates that miR-222 blockade does not merely slow cell growth but actively enhances cell death under cytotoxic stress. This is important because apoptosis failure is a central feature of AML treatment resistance and remains one of the main reasons leukemic cells survive despite exposure to standard chemotherapy [8, 9, 19]. The magnitude of apoptosis induction observed here therefore supports a biologically meaningful interaction between miR-222 suppression and cytarabine exposure.

The qPCR data further revealed a transcriptional profile consistent with activation of the intrinsic mitochondrial apoptotic pathway. Upregulation of *BAX* mRNA alongside downregulation of *BCL-2* and *MCL-1* provides a plausible molecular explanation for the increased Annexin V positivity seen in the combination group [20, 25]. In AML, the balance between pro-apoptotic and anti-apoptotic BCL-2 family members is critically important for determining whether chemotherapy exposure culminates in cell death or survival, and this has become even more evident with the growing

therapeutic relevance of apoptosis-targeted strategies [8, 9]. Our results fit well with that framework and suggest that miR-222 inhibition may lower the apoptotic threshold sufficiently to enhance cytarabine responsiveness.

At the same time, these findings should be interpreted with appropriate caution. We emphasize that the present mechanistic data are based on mRNA expression, not direct protein or activity measurements. Because apoptosis biology is strongly regulated at post-transcriptional and post-translational levels, changes in *BAX*, *BCL-2*, or *MCL-1* transcripts do not by themselves establish pathway execution. Definitive mechanistic conclusions will therefore require protein-level validation, including Western blotting for BAX, BCL-2, MCL-1, cleaved caspase-3, and PARP, as well as functional assays of caspase activation. Accordingly, the present qPCR data should be viewed as hypothesis-supporting rather than mechanistically conclusive [8, 9, 19].

Another notable finding was the marked reduction in *C-KIT* mRNA, particularly in cells receiving both LNA-anti-miR-222 and cytarabine. *C-KIT* is an established growth and survival regulator in AML, and its overexpression has been associated with leukemic progression and adverse biology [21–24]. The present results suggest that miR-222 may help maintain *C-KIT*-dependent survival signaling in HL-60 cells and that its inhibition may weaken this pathway sufficiently to increase cytarabine susceptibility. Although *WT1* showed only a modest decline and *CEBPA* remained relatively stable, the strong suppression of *C-KIT* points to a survival-signaling effect that may be especially relevant in AML cells with dependence on receptor-mediated proliferative pathways [22, 24].

The present results also align with earlier work from the same experimental line showing that miR-222 inhibition in HL-60 cells is associated with increased apoptosis and downregulation of *C-KIT*, thereby supporting internal consistency across related models [18]. In addition, recent evidence has suggested that miR-222-3p can directly enhance AML cell proliferation and suppress apoptosis through prosurvival signaling pathways, further supporting the biologic plausibility of the current findings [4]. Taken together, these observations strengthen the rationale for viewing miR-222 as more than a passive biomarker and instead as a potentially actionable regulator of leukemic cell fitness.

From a translational perspective, these findings support miR-222 as a promising adjunctive target in AML. Cytarabine remains widely used in induction and salvage regimens, yet drug resistance remains a major barrier to cure [1]. A strategy that sensitizes leukemic cells to cytarabine without requiring an entirely new therapeutic backbone may therefore be clinically valuable. The observation that the combination effect was robust across multiple concentrations strengthens this interpretation, as it suggests that the effect is not highly sensitive to a single arbitrary dose choice. Moreover, the presence of a significant interaction term is consistent with the broader principle that combination studies should be evaluated quantitatively rather than inferred qualitatively from separate single-agent effects [7]. Although we did not perform a full Chou–Talalay combination-index analysis, the present results provide an appropriate preclinical basis for such follow-up work.

5 Limitations

This study has several important limitations. First, it was conducted in a single AML cell line (HL-60) and therefore cannot capture the full biologic heterogeneity of AML, which is now recognized as a molecularly diverse disease encompassing multiple cytogenetic, mutational, and transcriptional subtypes [10, 21]. Second, and most critically, all mechanistic conclusions are based on mRNA expression data without protein-level validation. The intrinsic apoptosis pathway is tightly regulated at the post-transcriptional and post-translational levels; therefore, changes in transcript abundance do not always correlate with changes in functional protein levels or biologic activity [8, 19]. Future work should include Western blotting for BAX, BCL-2, MCL-1, cleaved caspase-3, PARP, and C-KIT, along with functional assays such as caspase activity measurements.

Third, while the concentration-response matrix and two-way ANOVA support an interaction between the two agents, we did not calculate formal combination indices. A full synergy analysis using the Chou–Talalay method or related approaches would require more extensive fixed-ratio dose-response testing and should be included in future studies [7]. Fourth, the study did not examine off-target effects of

LNA-anti-miR-222 or potential effects on non-malignant hematopoietic cells. Fifth, the translational implications remain preliminary until the findings are tested in additional AML cell lines, primary patient samples, and in-vivo models. These next steps will be particularly important for determining whether the observed combination effect extends beyond HL-60 cells and whether it retains activity in cytarabine-resistant settings, which are of major clinical importance [1, 25].

Overall, the present data support a model in which miR-222 contributes to AML cell survival and relative chemotherapy tolerance, and in which its inhibition lowers the threshold for cytarabine-induced apoptosis. Within the limitations of an in-vitro, mRNA-focused design, this study provides a rational preclinical basis for further investigation of miR-222-directed combination therapy in AML.

6 Conclusion

Blockade of miR-222 enhanced the pro-apoptotic and anti-proliferative effects of cytarabine in HL-60 acute myeloid leukemia cells across a range of concentrations. The combination treatment produced the greatest decrease in viability, the highest apoptosis rate, and was associated with increased *BAX* mRNA, decreased *BCL-2* and *MCL-1* mRNA, and markedly suppressed *C-KIT* mRNA. These findings support miR-222 as a potential chemosensitizing target in AML and provide a rationale for further preclinical evaluation, including protein-level mechanistic studies and in-vivo efficacy testing.

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