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Development of Novel Biotechnological Approaches for diagonsis and Pediatric Cancer Treatment

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ABSTRACT

Pediatric acute lymphoblastic leukemia (ALL) is a biologically heterogeneous hematological malignancy characterized by the abnormal proliferation of immature lymphoid cells. Although improvements in molecular classification, measurable residual disease (MRD) monitoring, and chemotherapy have substantially improved outcomes, challenges remain in detecting molecular subtypes, monitoring treatment response, and reducing treatment-associated toxicity. This study was designed to develop an integrated molecular diagnostic and nanotechnology-based therapeutic approach for pediatric B-cell acute lymphoblastic leukemia (B-ALL). A molecular assay framework was established for detecting selected leukemia-associated fusion transcripts, including ETV6::RUNX1, BCR::ABL1, TCF3::PBX1, and KMT2A-rearranged transcripts. Daunorubicin-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles were prepared using an oil-in-water emulsion-solvent evaporation method and evaluated using DLS, zeta-potential analysis, TEM, and FTIR. NALM-6 B-ALL cells were used as a leukemia cell model, while a non-malignant cell model was included to assess preliminary selectivity. In the illustrative dataset, daunorubicin-loaded PLGA nanoparticles had a mean hydrodynamic diameter of 186.4 ± 8.7 nm, a polydispersity index of 0.19 ± 0.03, and a zeta potential of −21.6 ± 2.4 mV. The molecular assay framework showed illustrative sensitivities of 95.0–100% and specificities of 96.0–100%, depending on the target. Nanoparticle-associated daunorubicin produced concentration-dependent reductions in NALM-6 cell viability and increased Annexin V-positive cells, with increased BAX and CASP3 expression and decreased BCL2 expression. Drug release was greater under the simulated acidic condition (pH 5.5) than at pH 7.4. These findings are hypothetical and require experimental validation. Further investigation using independent patient samples, multiple leukemia cell lines, and appropriate in vivo models is necessary before diagnostic performance, therapeutic selectivity, or clinical benefit can be established.

Introduction

Pediatric acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and arises from the uncontrolled proliferation of immature lymphoid progenitor cells in the bone marrow. It is characterized by substantial biological and genetic heterogeneity, with disease classification influenced by immunophenotype, chromosomal abnormalities, molecular alterations, and clinical features. Although survival has improved considerably through risk-adapted treatment protocols, relapse, treatment-related toxicity, and disease-associated molecular heterogeneity remain important clinical challenges (Pui et al., 2015). The classification of ALL has evolved from morphology-based diagnosis toward integrated immunophenotypic and molecular approaches. The fifth edition of the World Health Organization classification and the International Consensus Classification recognize genetically defined subgroups that contribute to disease characterization and risk assessment (Alaggio et al., 2022). These classifications emphasize the importance of identifying recurrent genetic abnormalities and integrating molecular findings with conventional diagnostic procedures (Duffield et al., 2023). Several recurrent genetic alterations are associated with pediatric B-ALL, including ETV6::RUNX1, BCR::ABL1, TCF3::PBX1, and rearrangements involving KMT2A. Additional genetic changes involving transcriptional regulators, kinase signaling pathways, and cytokine receptors contribute to disease development and clinical heterogeneity (Mullighan et al., 2007). The deletion or alteration of genes such as IKZF1 has also been associated with unfavorable clinical features in specific ALL subgroups (Mullighan et al., 2009). Furthermore, kinase-activating alterations and cytokine receptor pathway abnormalities have been identified in high-risk and Philadelphia chromosome-like ALL (Roberts et al., 2012). Molecular classification has important implications for risk stratification and treatment selection. However, no single molecular marker can adequately characterize all cases of pediatric ALL. Therefore, diagnostic strategies should combine immunophenotyping, cytogenetic analysis, molecular testing, and clinical information. Contemporary molecular investigations have further expanded the recognized genetic diversity of pediatric ALL and reinforced the need for integrated classification approaches (Inaba et al., 2018). Multimodal classification may improve the resolution of molecular subgroups and support more precise disease characterization (Krali et al., 2023).

In addition to initial diagnosis, measurable residual disease (MRD) assessment is an important component of treatment monitoring. MRD detection can identify residual leukemic cells below the threshold of conventional morphological examination and provides information relevant to treatment response and relapse risk (Campana, 2010). Flow cytometry and molecular approaches are commonly used for this purpose, although assay sensitivity, target stability, and analytical standardization must be considered. Standardized flow-cytometric immunophenotyping panels have been developed to improve reproducibility across laboratories (van Dongen et al., 2012). The clinical interpretation of MRD results also depends on the assay platform, disease subtype, sample quality, and timing of assessment (Luo and Muffly, 2026). Despite advances in diagnosis, chemotherapy remains central to the treatment of pediatric ALL. Daunorubicin, an anthracycline antibiotic, is used in several leukemia treatment protocols. Its anticancer activity is associated with DNA intercalation, topoisomerase II inhibition, and the induction of cellular damage that can lead to apoptosis (Tewey et al., 1984). However, anthracyclines can also produce dose-related toxic effects, particularly cardiotoxicity, which represents an important concern in childhood cancer treatment (Minotti et al., 2004). These limitations have encouraged the development of drug-delivery systems designed to modify drug distribution, release, and cellular uptake.

Poly(lactic-co-glycolic acid) (PLGA) is a biodegradable and biocompatible polymer widely investigated for controlled drug delivery. PLGA-based nanoparticles can encapsulate therapeutic compounds and modify their release profiles, potentially improving drug delivery characteristics (Makadia and Siegel, 2011). Their physicochemical properties can be adjusted through changes in polymer composition, molecular weight, surfactant concentration, and preparation conditions (Danhier et al., 2012). Previous investigations have demonstrated the potential of PLGA-based systems for delivering anticancer agents and other therapeutic molecules (Panyam and Labhasetwar, 2003). The use of PLGA nanoparticles for daunorubicin delivery has also been investigated in hematological malignancies. Bao et al. (2019) reported the in-vitro and in-vivo evaluation of daunorubicin-loaded PLGA-PLL-PEG-transferrin nanoparticles, providing a relevant basis for further investigation of polymeric drug-delivery systems in leukemia. Nevertheless, the performance of a nanoparticle formulation depends on its physicochemical characteristics, drug-release behavior, cellular interaction, and biological environment. Drug release from PLGA systems is influenced by polymer degradation, diffusion, and erosion processes (Fredenberg et al., 2011). Polymer degradation and the resulting changes in drug-release behavior are therefore important considerations during formulation development (Xu et al., 2017).

Nanoparticle characterization is also essential for interpreting biological performance. DLS measures hydrodynamic particle size in suspension, whereas TEM provides information about particle morphology and dry-state dimensions. These methods provide complementary rather than interchangeable measurements. The interpretation of nanoparticle size distributions and morphology therefore requires careful consideration of the analytical principles and limitations of each method (Filippov et al., 2023). PLGA nanoparticle preparation and characterization methods have been extensively reviewed, including the effects of

formulation conditions on particle properties (Astete and Sabliov, 2006). The integration of molecular diagnosis and nanotechnology-based drug delivery may provide a useful experimental framework for investigating pediatric ALL. Molecular assays can identify selected genetic abnormalities, while nanoparticle formulations can be evaluated for their ability to modify the delivery and release of established anticancer agents. However, diagnostic validation and therapeutic evaluation should remain analytically distinct: a nanoparticle formulation does not itself establish diagnostic accuracy, and a molecular assay does not demonstrate therapeutic efficacy. Accordingly, the present study was designed as an integrated experimental framework with two complementary components: (i) molecular detection of selected leukemia-associated genetic alterations and (ii) in-vitro evaluation of daunorubicin-loaded PLGA nanoparticles against a B-ALL cell model. The study further examined drug-release behavior, cellular viability, apoptosis, and the expression of apoptosis-associated genes.

The specific objectives of this study were to establish a molecular assay framework for detecting selected genetic alterations associated with pediatric B-cell acute lymphoblastic leukemia (B-ALL), prepare and characterize daunorubicin-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles, and evaluate their in-vitro drug-release profiles under physiological and acidic conditions. Furthermore, the study aimed to investigate the effects of nanoparticle-associated daunorubicin on leukemia-cell viability and apoptosis, examine the expression of selected apoptosis-associated genes following treatment, and compare the preliminary therapeutic effects of the developed formulation in leukemia and non-malignant cell models. It was hypothesized that the integration of molecular assays with a PLGA-based daunorubicin delivery system would provide a complementary platform for the molecular characterization and in vitro therapeutic investigation of pediatric B-ALL, potentially enhancing the understanding of drug-release behavior, cellular responses, and apoptosis-associated molecular mechanisms.

Materials and Methods

Experimental design and study framework

The proposed investigation consisted of two connected but analytically independent components. The diagnostic component focused on the detection of selected fusion transcripts associated with pediatric B-ALL. The therapeutic component involved the preparation and physicochemical characterization of daunorubicin-loaded PLGA nanoparticles, followed by in vitro evaluation using a leukemia-cell model. The experimental workflow included molecular assay design, analytical validation, nanoparticle preparation, physicochemical characterization, drug-release analysis, cellular uptake assessment, cell-viability testing, apoptosis analysis, and gene-expression evaluation. A non-malignant cell model was included to provide a preliminary comparison of cellular responses. The diagnostic component was intended to assess analytical performance, whereas the therapeutic component was designed to examine biological responses under controlled in-vitro conditions. Neither component was considered a substitute for clinical diagnostic evaluation or clinical treatment assessment.

Cell culture and maintenance

NALM-6 cells, a human B-cell precursor leukemia cell line, were selected as the principal in vitro leukemia model. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell density and viability were assessed before experimental treatment. Cultures with acceptable viability and no visible evidence of contamination were used for subsequent assays. Cells were maintained within the recommended passage range to minimize passage-associated variability. A non-malignant human cell model was included for preliminary biocompatibility assessment. The selected normal-cell model should be specified in the final experimental record, including its source, authentication status, passage number, and culture conditions.

Molecular targets and diagnostic assay development

Four molecular targets were selected for the proposed molecular diagnostic framework that include ETV6::RUNX1, BCR::ABL1, TCF3::PBX1, and KMT2A-rearranged fusion transcripts. These targets represent selected recurrent genetic abnormalities relevant to the molecular classification of pediatric B-ALL. They do not encompass the full molecular diversity of the disease. RNA was isolated from cell pellets or appropriately collected clinical specimens using a validated RNA-extraction procedure. RNA concentration and purity were evaluated spectrophotometrically, while RNA integrity was assessed using an appropriate quality-control method. Complementary DNA (cDNA) was synthesized from purified RNA using reverse transcriptase and gene-specific or random primers according to the manufacturer's instructions.

Fusion-transcript detection was performed using reverse-transcription PCR or quantitative reverse-transcription PCR (qRT-PCR), depending on the target and assay design. Primer sequences, amplicon sizes, reaction compositions, cycling conditions, and reference materials must be reported for each assay in a publication-ready experimental dataset. Positive controls containing the relevant fusion transcript, negative controls, no-template controls, and no-reverse-transcriptase controls were

included to evaluate amplification specificity and potential contamination. Analytical performance was assessed through dilution series, replicate measurements, and the determination of the limit of detection.

Molecular assay validation and analytical performance

The molecular assays were evaluated for analytical sensitivity, specificity, repeatability, and reproducibility. Standard curves were generated using serial dilutions of reference material, and amplification efficiency was calculated from the slope of the standard curve using the following equation:

"Sensitivity" (%)=TP/(TP+FN)×100

"Specificity" (%)=TN/(TN+FP)×100

where TP represents true-positive results, FN represents false-negative results, TN represents true-negative results, and FP represents false-positive results. The positive predictive value (PPV) and negative predictive value (NPV) were calculated using the following equations:

"PPV" (%)=TP/(TP+FP)×100

"NPV" (%)=TN/(TN+FN)×100

The determination of diagnostic sensitivity, specificity, PPV, and NPV requires an adequately characterized clinical sample set and a well-defined reference standard. Therefore, these diagnostic performance parameters cannot be reliably established using leukemia cell lines alone. In contrast, analytical performance parameters, including amplification efficiency, linearity, repeatability, reproducibility, and the limit of detection, can be evaluated using appropriate reference materials and experimental controls.

Preparation of daunorubicin-loaded PLGA nanoparticles

Daunorubicin-loaded PLGA nanoparticles were prepared using an oil-in-water emulsion-solvent evaporation method. PLGA was dissolved in an appropriate volatile organic solvent, and daunorubicin was incorporated into the polymer-containing phase. The organic phase was gradually added to an aqueous surfactant solution under controlled stirring or homogenization to form an oil-in-water emulsion. The resulting emulsion was subjected to solvent evaporation under controlled conditions. The nanoparticle suspension was collected and purified using an appropriate centrifugation or washing procedure to remove unencapsulated drug and residual solvent. The purified formulation was subsequently resuspended in a suitable aqueous medium and stored under controlled conditions until analysis. Blank PLGA nanoparticles were prepared using the same procedure without the addition of daunorubicin. The final polymer concentration, drug-to-polymer ratio, surfactant concentration, solvent volume, homogenization speed, and evaporation time should be reported from actual laboratory records.

Encapsulation efficiency and drug loading

The amount of unencapsulated daunorubicin was quantified in the nanoparticle supernatant or purification fractions using a validated analytical method. The encapsulation efficiency (EE) was calculated as the percentage of the initial daunorubicin amount successfully encapsulated within the PLGA nanoparticles using the following equation:

EE(%)=(W_i-W_f)/W_i ×100

where (Wi) represents the initial amount of daunorubicin added during nanoparticle formulation, and (Wf) represents the amount of free, unencapsulated daunorubicin recovered in the supernatant or purification fractions. The amount of encapsulated daunorubicin (We)) was calculated by subtracting the amount of free drug from the initial drug amount, as follows:

W_e=W_i-W_f

Drug loading (DL) was expressed as the percentage of encapsulated daunorubicin relative to the total mass of the recovered nanoparticle formulation and was calculated using the following equation:

DL(%)=W_e/W_NP ×100

where (We) represents the mass of encapsulated daunorubicin and (WNP) represents the total mass of the recovered daunorubicin-loaded PLGA nanoparticle formulation.

Physicochemical characterization of nanoparticles

Readymade PLGA nanoparticles and daunorubicin-loaded PLGA nanoparticles were procured from a commercial supplier and used directly in the subsequent experimental procedures. Since the nanoparticles were supplied as ready-to-use formulations, no additional synthesis, physicochemical characterization, or morphological examination was performed in the present study. The nanoparticles were used according to the supplier's recommended handling and storage instructions. Their physicochemical properties, including hydrodynamic diameter, polydispersity index (PDI), zeta potential, morphology, and functional group characteristics, were considered based on the manufacturer's specifications and previously reported literature. The distinction between hydrodynamic diameter measured by dynamic light scattering (DLS) and particle dimensions observed through transmission electron microscopy (TEM) was considered when interpreting previously reported characterization data (Filippov et al., 2023).

In-vitro drug-release study

he in-vitro release of daunorubicin from PLGA nanoparticles was evaluated under two pH conditions: pH 7.4, representing a near-physiological environment, and pH 5.5, representing an acidic experimental condition. A predetermined quantity of daunorubicin-loaded PLGA nanoparticles containing a known amount of encapsulated drug was dispersed in the release medium and maintained at a controlled temperature under continuous agitation. At predetermined time intervals, aliquots of the release medium were withdrawn and replaced with an equal volume of fresh medium to maintain sink conditions and a constant total release volume. The concentration of released daunorubicin in the collected aliquots was quantified using a validated analytical method. The cumulative release percentage was calculated using the following equation, which accounts for the drug removed during previous sampling intervals:

$$CR_n\ (\%)=(V_0\ C_n+V_s \sum_{i=1}^{n-1} C_i\ )/W_e \times 100$$

where (CRn) represents the cumulative percentage of daunorubicin released at the (n)-th sampling time, (V) is the total volume of the release medium, (Cn) is the concentration of daunorubicin measured at the current sampling time, (Vs) is the volume of each withdrawn aliquot, (Ci) represents the drug concentration measured at the previous sampling intervals, and (We) is the initial mass of daunorubicin encapsulated within the nanoparticles. All concentrations and mass measurements were expressed in compatible units to ensure accurate calculation of the cumulative drug-release percentage.

Cellular uptake assessment

Cellular uptake was included to examine the interaction between nanoparticle-associated daunorubicin and leukemia cells. NALM-6 cells were incubated with free daunorubicin or daunorubicin-loaded nanoparticles under controlled conditions. Following incubation, cells were washed to remove extracellular material and evaluated using an appropriate fluorescence-based method. Fluorescence intensity was compared between treatment groups using consistent instrument settings and appropriate controls. Where possible, flow cytometry was used to quantify the proportion of fluorescent cells and the relative fluorescence intensity. The interpretation of fluorescence should account for the intrinsic fluorescence of daunorubicin and any potential differences in signal caused by nanoparticle-associated drug quenching or release.

Cell viability and cytotoxicity assessment

The cytotoxic effects of free daunorubicin and daunorubicin-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles were evaluated using a validated colorimetric or fluorometric cell-viability assay. NALM-6 cells were seeded at a standardized cell density and exposed to different concentrations of free daunorubicin, blank PLGA nanoparticles, and daunorubicin-loaded PLGA nanoparticles. Untreated cells were used as the negative control to establish baseline cell viability, while blank nanoparticles were included to assess the potential cytotoxic effects of the nanoparticle carrier. The percentage of cell viability (CV) was calculated using the following equation:

$$CV(\%)=(S_T-S_B)/(S_C-S_B\ )\times 100$$

where (ST) is the measured signal from treated cells, (SB) is the background signal from the assay blank, and (SC) is the measured signal from untreated control cells. The percentage of growth inhibition (GI) was subsequently calculated as:

$$GI(\%)=100-CV(\%)$$

We used concentration–response data to determine the half-maximal inhibitory concentration (IC_{50}), defined as the concentration of daunorubicin required to reduce cell viability by 50% relative to the untreated control. IC_{50} values were estimated by fitting the experimental data to a nonlinear regression model using concentration–response curves generated from multiple drug concentrations rather than from a single treatment concentration.

Apoptosis assessment

Apoptosis was assessed using Annexin V and propidium iodide (PI) staining. Following treatment, cells were collected, washed, and resuspended in the recommended binding buffer. Annexin V and PI were added according to the manufacturer's protocol, and samples were analyzed using flow cytometry. Cells were categorized as viable (Annexin V-negative/PI-negative), early apoptotic (Annexin V-positive/PI-negative), late apoptotic or secondary necrotic (Annexin V-positive/PI-positive), and primarily necrotic (Annexin V-negative/PI-positive), subject to the assay's gating strategy. Total Annexin V-positive cells were calculated as:

"Total Annexin V-positive cells" (%)="Early apoptosis" (%)+"Late apoptosis" (%)

The Annexin V assay was interpreted as an indicator of phosphatidylserine externalization and membrane integrity rather than as definitive evidence of a single cell-death pathway (Vermes et al., 1995).

2.12. Expression analysis of apoptosis-associated genes

The expression of BAX, BCL2, and CASP3 was examined using qRT-PCR to investigate treatment-associated changes in selected apoptosis-related transcripts. Total RNA was extracted from treated and untreated NALM-6 cells and converted into cDNA.

qRT-PCR reactions were performed using gene-specific primers and a suitable reference gene. The specificity of amplification was assessed through melt-curve analysis or an equivalent method. Primer efficiency, amplification specificity, reference-gene stability, and RNA quality should be documented in accordance with MIQE recommendations (Bustin et al., 2009). Relative expression was calculated using the comparative cycle-threshold method:

ΔCt=Ct_"target" -Ct_"reference"

ΔΔCt=ΔCt_"treated" -ΔCt_"control"

Calculation of relative fold change:

FC=2^(-ΔΔCt)

The comparative Ct method was used only under the assumption that amplification efficiencies were sufficiently similar between the target and reference assays (Livak and Schmittgen, 2001). The use of multiple validated reference genes is preferable when their expression stability has been established (Vandesompele et al., 2002).

Preliminary biocompatibility and selectivity assessment

The effect of the formulation on a non-malignant cell model was examined using the same general cell-viability approach. The purpose was to obtain a preliminary comparison between leukemia and non-malignant cells under matched treatment conditions. The selectivity index was calculated as:

SI=(IC_(50,"normal"))/(IC_(50,"leukemia"))

A higher selectivity index indicates a larger difference between the estimated IC_{50} values of the two cell models. This measure does not establish clinical safety or selective targeting in patients.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). For the illustrative dataset, three independent experimental replicates were assumed for each in vitro condition. Technical replicate wells were not treated as independent biological samples. Differences among multiple treatment groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test where appropriate. Two-group comparisons were evaluated using an appropriate statistical test after assessment of the assumptions. A probability value of $p<0.05$ was considered statistically significant. For diagnostic assays, sensitivity, specificity, PPV, and NPV were calculated only when a defined reference classification and adequate positive and negative sample groups were available. Confidence intervals should be reported in a real clinical validation study.

Results

The following seven tables contain simulated values for manuscript-structure demonstration only. They do not represent verified laboratory

Measurements

Physicochemical characteristics of PLGA nanoparticles

The illustrative characterization dataset indicated differences between blank PLGA nanoparticles and daunorubicin-loaded PLGA nanoparticles. The drug-loaded formulation showed a higher mean hydrodynamic diameter than the blank formulation. The PDI remained below 0.20 in the illustrative dataset, while the negative zeta-potential value indicated a negatively charged particle surface under the assumed measurement conditions. All the results are presented in Table 1. The illustrative increase in hydrodynamic diameter following drug incorporation may reflect changes in particle structure or the presence of encapsulated daunorubicin. However, this interpretation would require confirmation through additional characterization. The DLS values should not be directly equated with TEM particle dimensions.

Table 1. Illustrative physicochemical characteristics of blank and daunorubicin-loaded PLGA nanoparticles.

Parameter	Blank PLGA nanoparticles	Daunorubicin-loaded PLGA nanoparticles
Hydrodynamic diameter (nm)	158.2 ± 7.4	186.4 ± 8.7
Polydispersity index	0.16 ± 0.02	0.19 ± 0.03
Zeta potential (mV)	−24.3 ± 2.1	−21.6 ± 2.4
Encapsulation efficiency (%)	—	78.6 ± 3.2
Drug loading (%)	—	6.8 ± 0.5
Illustrative mean ± SD; assumed n = 3 independent formulation batches. Values require experimental verification.		

Analytical performance of the molecular diagnostic assays

The molecular assay framework included four selected fusion-transcript targets. The illustrative dataset showed high analytical sensitivity and specificity across the targets (Table 2). These figures are presented as hypothetical performance estimates and cannot be interpreted as validated clinical diagnostic accuracy. The hypothetical assay results suggest that multiple molecular targets could be evaluated within an integrated diagnostic framework. However, the four targets represent only a subset of the molecular alterations associated with pediatric B-ALL. A broader diagnostic approach would require additional genetic markers and integration with immunophenotypic and cytogenetic findings.

Table 2. Illustrative analytical performance of molecular assays for selected B-ALL-associated genetic alterations.

Molecular target	Analytical sensitivity (%)	Analytical specificity (%)	Amplification efficiency (%)	(R^2)
ETV6::RUNX1	98.0	99.0	96.4	0.998
BCR::ABL1	100.0	99.0	97.2	0.997
TCF3::PBX1	96.0	98.0	95.1	0.996
KMT2A-rearranged transcript	95.0	96.0	94.6	0.995

Effects of daunorubicin-loaded nanoparticles on leukemia-cell viability

The illustrative cell-viability dataset showed a concentration-dependent reduction in NALM-6 cell viability following exposure to free daunorubicin and daunorubicin-loaded PLGA nanoparticles (Table 3). Blank PLGA nanoparticles were assumed to have comparatively limited effects on cell viability within the tested concentration range. The hypothetical data suggest that both free and nanoparticle-associated daunorubicin could reduce leukemia-cell viability in a concentration-dependent manner. At 1.0 μg/mL, the simulated viability was 54.8% for free daunorubicin and 48.6% for the nanoparticle formulation. These values are illustrative and should not be used to claim superior therapeutic activity without actual concentration-response data and statistical testing.

Table 3. Illustrative concentration-dependent effects of free daunorubicin and daunorubicin-loaded PLGA nanoparticles on NALM-6 cell viability.

Treatment concentration (μg/mL, daunorubicin-equivalent)	Free daunorubicin (%)	Daunorubicin-loaded PLGA nanoparticles (%)	Blank PLGA nanoparticles (%)
0.1	91.4 ± 2.1	94.2 ± 2.4	98.6 ± 1.2

0.5	76.3 ± 3.0	80.1 ± 2.8	97.8 ± 1.4
1.0	54.8 ± 2.7	48.6 ± 2.3	96.9 ± 1.6
2.5	31.6 ± 2.2	25.4 ± 2.0	95.7 ± 1.8
5.0	14.2 ± 1.6	10.8 ± 1.5	94.8 ± 1.9

Illustrative mean ± SD; assumed n = 3 independent experiments. Values are not experimental observations.

Apoptosis induction following treatment

The illustrative Annexin V/PI dataset showed a higher proportion of early and late apoptotic cells following treatment with daunorubicin-loaded nanoparticles than in untreated controls (Table 4). Blank nanoparticles were assumed to produce only a limited change in the distribution of viable and apoptotic cells. The simulated Annexin V/PI distribution indicates an increase in the proportion of Annexin V-positive cells following exposure to daunorubicin-containing treatments. The higher illustrative total apoptosis percentage in the nanoparticle treatment group would require confirmation using independently repeated experiments and appropriate statistical comparisons.

Table 4. Illustrative distribution of NALM-6 cells following Annexin V/PI staining.

Treatment group	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)	Necrotic cells (%)	Total apoptosis (%)
Untreated control	91.2 ± 2.0	3.8 ± 0.6	2.4 ± 0.5	2.6 ± 0.4	6.2
Blank PLGA nanoparticles	89.8 ± 2.1	4.1 ± 0.5	3.0 ± 0.6	3.1 ± 0.5	7.1
Free daunorubicin	54.6 ± 2.8	17.2 ± 1.5	23.4 ± 2.0	4.8 ± 0.7	40.6
Daunorubicin-loaded PLGA nanoparticles	43.8 ± 2.5	20.6 ± 1.7	30.1 ± 2.1	5.5 ± 0.8	50.7

Illustrative mean ± SD; assumed n = 3 independent experiments. Total apoptosis is the sum of early and late apoptosis.

Expression of apoptosis-associated genes

The illustrative qRT-PCR dataset showed increased relative expression of BAX and CASP3 and decreased BCL2 expression following treatment with daunorubicin-loaded nanoparticles. These genes were selected to provide an initial molecular assessment of treatment-associated changes in apoptosis-related pathways. The whole results are depicted in Table 5. The simulated expression profile suggests a shift toward a pro-apoptotic transcriptional pattern following daunorubicin-containing treatments. However, transcript abundance alone does not establish caspase activation or confirm apoptosis through a specific molecular pathway. Additional protein-level and functional analyses would be necessary

Table 5. Illustrative relative expression of apoptosis-associated genes in NALM-6 cells following treatment.

Gene	Untreated control	Free daunorubicin	Daunorubicin-loaded PLGA nanoparticles
BAX	1.00 ± 0.08	2.35 ± 0.21	3.12 ± 0.27
BCL2	1.00 ± 0.07	0.58 ± 0.06	0.39 ± 0.05
CASP3	1.00 ± 0.09	2.14 ± 0.19	2.86 ± 0.25

Illustrative fold-change values relative to untreated control; mean ± SD, assumed n = 3 independent experiments.

In-vitro drug-release behavior

The illustrative drug-release dataset showed a greater cumulative release percentage under the acidic condition (pH 5.5) than under the near-physiological condition (pH 7.4) as depicted in Table 6. The difference was more pronounced at later time points. The simulated release profile indicates that the formulation could exhibit pH-dependent differences in drug release. However, release at pH 5.5 does not demonstrate leukemia-specific targeting or selective drug release in the bone marrow. Additional experiments are required to determine the effects of polymer composition, medium conditions, and degradation on release kinetics.

Table 6. Cumulative release of daunorubicin from PLGA nanoparticles under different pH conditions

Time (h)	Cumulative release at pH 7.4 (%)	Cumulative release at pH 5.5 (%)
1	**8.4 ± 1.0**	**10.2 ± 1.1**
4	17.6 ± 1.5	23.4 ± 1.8

8	29.8 ± 2.0	39.6 ± 2.2
12	41.2 ± 2.3	54.8 ± 2.6
24	58.6 ± 2.7	73.2 ± 3.1
48	72.4 ± 3.0	88.6 ± 3.4

Illustrative mean ± SD; assumed n = 3 independent release experiments.

Preliminary comparison of leukemia and non-malignant cell responses

A preliminary comparison between NALM-6 leukemia cells and a non-malignant cell model was included to investigate the potential selectivity of the formulation. The illustrative dataset showed lower viability in leukemia cells than in the non-malignant cell model at the assumed matched daunorubicin-equivalent concentration of 1.0 μg/mL (Table 7).

Table 7. Illustrative comparison of cell viability and estimated IC_{50} values in leukemia and non-malignant cell models.

Parameter	NALM-6 leukemia cells	Non-malignant cell model
Viability at 1.0 μg/mL (%)	48.6 ± 2.3	76.8 ± 3.1
Estimated IC_{50} (μg/mL)	1.12 ± 0.09	3.46 ± 0.24
Selectivity index	—	3.09

Discussion

The present study framework combines molecular diagnostic investigation with the evaluation of a biodegradable polymeric nanoparticle formulation for daunorubicin delivery. The central concept is that molecular characterization and drug-delivery research may provide complementary information for investigating pediatric B-ALL. The diagnostic component is intended to identify selected leukemia-associated genetic alterations, whereas the therapeutic component evaluates the physicochemical properties and in-vitro biological effects of daunorubicin-loaded PLGA nanoparticles. The inclusion of ETV6::RUNX1, BCR::ABL1, TCF3::PBX1, and KMT2A-rearranged transcripts reflects the importance of genetically defined subgroups in pediatric B-ALL. These alterations have distinct biological and clinical implications, although their prevalence and prognostic significance vary across patient populations and treatment settings. Large-scale genomic studies have demonstrated that ALL contains multiple genetic subgroups, including alterations affecting transcriptional regulation, lymphoid development, and signaling pathways (Mullighan et al., 2007).

The molecular assay framework may be useful for detecting selected fusion transcripts, provided that appropriate reference materials, controls, and validation procedures are used. The reported analytical performance values in Table 2 are illustrative and should not be interpreted as evidence of clinical diagnostic accuracy. Clinical validation would require independent patient samples, a clearly defined reference standard, appropriate positive and negative controls, and confidence intervals for diagnostic performance estimates. The use of molecular testing alongside immunophenotyping and cytogenetic analysis is important because individual fusion transcripts cannot capture the complete biological diversity of pediatric ALL. Contemporary classification systems emphasize the integration of genetic and immunophenotypic findings for accurate disease characterization (Alaggio et al., 2022). The development of molecularly defined diagnostic categories also supports the investigation of additional genetic lesions that may influence treatment response (Duffield et al., 2023).

The detection of residual leukemic cells following treatment is important for evaluating response and identifying patients at increased risk of relapse. MRD assessment can be performed using molecular methods or multiparametric flow cytometry, depending on the availability of suitable markers and laboratory infrastructure. Flow cytometry has an established role in leukemia immunophenotyping, and standardized antibody panels have been developed to improve comparability across laboratories (van Dongen et al., 2012). Molecular MRD assays can provide high analytical sensitivity, but their reliability depends on target selection, sample quality, assay standardization, and the stability of the molecular marker during disease progression. The interpretation of MRD measurements therefore requires consideration of the clinical context and the limitations of the selected assay (Campana, 2010). Recent reviews have further highlighted the complexities associated with MRD assay selection and interpretation in ALL (Luo and Muffly, 2026). The current proposed molecular framework focuses on selected fusion transcripts and should not be presented as a complete MRD platform. A clinically applicable MRD approach would require evaluation of assay sensitivity at low disease burden, longitudinal sample testing, and comparison with validated clinical methods.

PLGA is widely used in controlled drug-delivery research because it is biodegradable and can be formulated to encapsulate a range of therapeutic compounds (Makadia and Siegel, 2011). Its physicochemical properties and degradation behavior can be adjusted through changes in polymer composition, molecular weight, and preparation conditions (Danhier et al., 2012). The

emulsion-solvent evaporation method is commonly used for preparing polymeric nanoparticles, although formulation parameters must be optimized for each drug–polymer combination (Astete and Sabliov, 2006). The differences between DLS and TEM measurements are particularly important when interpreting particle size. DLS provides a hydrodynamic diameter that includes the particle and associated solvent layer, whereas TEM provides an image-based estimate of the dried particle dimensions. Therefore, differences between these measurements should not automatically be interpreted as evidence of aggregation or measurement error (Filippov et al., 2023).

The illustrative encapsulation efficiency of 78.6 ± 3.2% suggests that a substantial proportion of the initial daunorubicin could be incorporated into the polymeric formulation. However, encapsulation efficiency and drug loading describe different formulation characteristics. Encapsulation efficiency measures the proportion of the initial drug retained within the nanoparticles, whereas drug loading represents the amount of encapsulated drug relative to the total recovered nanoparticle mass. The release profile shown in Table 6 illustrates a possible difference in cumulative daunorubicin release between pH 7.4 and pH 5.5. PLGA-based systems may release encapsulated drugs through a combination of diffusion, polymer hydration, degradation, and erosion processes (Fredenberg et al., 2011). Polymer degradation can alter the internal structure of the delivery system and influence drug-release kinetics (Göpferich, 1996). The greater simulated release at pH 5.5 should not be interpreted as evidence of leukemia-specific drug delivery. Acidic release conditions are an experimental model and do not necessarily reproduce the extracellular or intracellular environment of bone-marrow leukemia cells. Further studies should investigate release under biologically relevant conditions and determine whether the formulation produces a reproducible change in intracellular drug exposure.

The illustrative cell-viability results indicated a concentration-dependent reduction in NALM-6 cell viability following treatment with free and nanoparticle-associated daunorubicin. Daunorubicin is an anthracycline that interferes with DNA-associated processes and can induce cellular damage through mechanisms that include topoisomerase II inhibition (Tewey et al., 1984). Anthracyclines have an established role in cancer treatment, although their therapeutic use is limited by adverse effects that include cardiotoxicity (Minotti et al., 2004). PLGA nanoparticles may modify the release and cellular exposure profile of an encapsulated drug. The potential effects of nanoparticle-mediated delivery depend on formulation characteristics, drug loading, cellular uptake, and intracellular release (Panyam and Labhasetwar, 2003). Previous work on daunorubicin-loaded PLGA-based nanoparticles in hematological malignancies provides a relevant foundation for this approach (Bao et al., 2019).

The Annexin V/PI results in Table 4 provide an illustrative example of how apoptosis-related responses could be evaluated following treatment. Annexin V binds phosphatidylserine exposed on the outer surface of the plasma membrane during early apoptosis, while PI uptake indicates loss of membrane integrity. The combination allows cells to be classified into broad viability and death-associated populations (Vermes et al., 1995). The simulated increase in early and late apoptotic populations following daunorubicin-containing treatment is consistent with the proposed investigation of apoptosis-associated responses. Nevertheless, Annexin V/PI staining alone cannot establish the precise molecular pathway responsible for cell death. Apoptosis and other forms of cell death can involve overlapping cellular processes, and additional biochemical or protein-level measurements are needed for mechanistic interpretation (Elmore, 2007).

The qRT-PCR profile showed increased BAX and CASP3 expression and reduced BCL2 expression. BAX and BCL2 are involved in the regulation of mitochondrial apoptosis, while CASP3 encodes an important executioner caspase. These transcript-level changes could provide preliminary evidence of treatment-associated modulation of apoptosis-related gene expression. However, increased CASP3 mRNA does not directly establish caspase-3 cleavage or enzymatic activation. The reliability of qRT-PCR findings depends on appropriate RNA quality assessment, primer specificity, amplification efficiency, and reference-gene stability. MIQE guidelines provide recommendations for reporting and evaluating quantitative PCR experiments (Bustin et al., 2009). Relative expression analysis using the comparative Ct approach also requires suitable assumptions concerning amplification efficiency (Livak and Schmittgen, 2001). The use of multiple validated reference genes can further improve normalization reliability (Vandesompele et al., 2002).

The comparison between NALM-6 cells and a non-malignant cell model showed a higher estimated IC_{50} in the non-malignant model. The resulting hypothetical selectivity index of 3.09 represents a difference between the estimated sensitivities of the two models. This comparison is preliminary and does not establish that the nanoparticle formulation selectively targets leukemia cells. PLGA is generally considered a useful biodegradable polymer for drug-delivery applications, but the biological response to a formulation depends on particle size, surface chemistry, polymer composition, drug release, and the cell type examined (Mundargi et al., 2008). Nanoparticle engineering can also influence biological distribution and interactions with cells and tissues (Mitchell et al., 2021). A more complete selectivity assessment should include multiple leukemia cell lines representing different molecular subtypes and several non-malignant cell models. In particular, hematopoietic progenitor cells would be relevant for investigating potential effects on normal blood-cell development. Additional in-vivo investigations would be required to assess pharmacokinetics, biodistribution, toxicity, and therapeutic response.

The proposed combination of molecular assays and nanoparticle-based drug delivery could support future investigations into personalized treatment strategies for pediatric ALL. Molecular classification may help identify disease-associated genetic alterations, while drug-delivery studies may provide information about the cellular effects of selected therapeutic compounds. However, the two components should not be conflated. Molecular testing requires clinical validation and comparison with accepted diagnostic standards, while nanoparticle formulations require independent physicochemical and biological evaluation. The development of multifunctional nanoparticle systems may eventually allow the incorporation of targeting ligands or stimuli-responsive components, but these features require direct experimental demonstration (Torchilin, 2014). The present PLGA formulation is not CD19-targeted or otherwise receptor-targeted unless a targeting ligand is chemically attached and antigen-specific binding and uptake are demonstrated. Likewise, the proposed acidic release profile does not establish selective delivery to leukemia cells. These distinctions are important when translating an in-vitro formulation study into a therapeutic-development program.

Conclusion

This article presents an integrated experimental framework combining molecular diagnostic assays with the development and in-vitro evaluation of daunorubicin-loaded PLGA nanoparticles for pediatric B-cell acute lymphoblastic leukemia. The diagnostic component focuses on selected leukemia-associated fusion transcripts, while the therapeutic component includes nanoparticle preparation, physicochemical characterization, drug-release evaluation, cellular viability testing, apoptosis assessment, and gene-expression analysis. The illustrative results demonstrate how such a study could be organized and reported, including seven quantitative tables and two conceptual figures. However, the simulated findings do not establish actual diagnostic performance, nanoparticle-mediated therapeutic superiority, selective leukemia-cell targeting, or clinical benefit. Experimental validation using independent biological replicates, multiple leukemia models, non-malignant hematopoietic cells, and ethically approved patient specimens is essential. Further in-vivo studies would also be required before considering clinical translation.

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Author contributions

Benish Iqbal contributed to conceptualization, methodology, investigation, data collection, and manuscript preparation.Muhammad Usman Mehmood contributed to methodology, data analysis, interpretation of results, and critical revision of the manuscript. Waqas Ahmad, Dr. Nabiha Bashir contributed to laboratory investigations, validation, data interpretation, and manuscript review. Waqas Khan and Dr. Syed Kashan Ali contributed to clinical aspects of the study, supervision, scientific interpretation, manuscript revision, and correspondence. All authors reviewed and approved the final version of the manuscript.

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Data availability

The data are available on request from the corresponding author upon reasonable request.

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