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The niosomal nelarabine as a promising nano combination for retinoblastoma treatment: an *in vitro* study—experimental research

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Introduction: Retinoblastoma (RB), the most commonly occurring intraocular malignancy among children globally, represents 3% of childhood cancers. In the current study, the authors aim to evaluate the effectiveness of a new formulation of nelarabine (niosomal nelarabine) on RB cancer cells.

Methods: Field emission scanning electron microscopy (FE-SEM) and dynamic light scattering (DLS) characterized the physical properties of nelarabine nanoparticles. After cultivation of the Y79 cell line, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was performed to determine IC50 of niosomal nelarabine (Nio-Nelarobine) and also the cytotoxicity of Nio-Nelarobine and doxorubicin against Y79 cell line was investigated. The level of apoptosis was assessed by flow cytometry in selected groups. Also, the PTEN/AKT/FOXO1 gene expression level was measured using qRT-PCR.

Results: Y79 cell lines were treated with Nio-Nelarobine and doxorubicin. The treatment resulted in a dose-dependent inhibition of Y79 cell viability. However, Nio-Nelarobine showed a higher inhibitory activity with a diameter of about 167 nm. Both Nio-Nelarobine and doxorubicin induced apoptosis in cells, but Nio-Nelarobine treatment resulted in a higher number of apoptotic cells than doxorubicin treatment. The qRT-PCR results showed that the treatment with Nio-Nelarobine and doxorubicin led to an increase in the expression of PTEN and FOXO1 genes, while decreasing the expression of the AKT gene. Furthermore, the statistical significance of these results was higher in the Nio-Nelarobine group than in the doxorubicin group.

Conclusions: Nio-Nelarobine may be a functional therapeutic combination for RB treatment. Further experimental and preclinical investigations are necessary to verify this impact in greater detail.

Keywords: apoptosis, experimental research, nelarabine, niosomes, retinoblastoma

Introduction

Retinoblastoma (RB) is the most commonly occurring intraocular malignancy among children globally, accounting for 3% of childhood cancers[1]. On average, diagnosis occurs before age 2, with 95% of patients under 5. Additionally, cancer risk is consistent among genders and races[2]. Additionally, more than 90% of RB cases occur in developing countries[3].

HIGHLIGHTS

- Treatment with Nio-Nelarobine and doxorubicin inhibited Y79 cell viability in a dose-dependent manner; however, Nio-Nelarobine showed higher inhibitory activity with a diameter of approximately 167 nm.
- Flow cytometry revealed that both Nio-Nelarobine and doxorubicin-induced Apoptosis in cells. However, Nio-Nelarobine treatment resulted in a higher number of apoptotic cells compared to doxorubicin treatment.
- Based on the qRT-PCR results, the treatment with Nio-Nelarobine and doxorubicin caused an increase in the expression of PTEN and FOXO1 genes while decreasing the expression of the AKT gene.
- The statistical significance of these results was higher in the Nio-Nelarobine group than in the doxorubicin group.

Various methods and strategies were developed to enhance and treat RB[4], and there are several treatment approaches available for RB, such as chemotherapy, focal therapies like laser therapy or cryotherapy, radiation therapy, and surgical interventions like enucleation (removal of the affected eye) or plaque radiotherapy. Depending on the extent of the tumor, the child's overall health, and the potential impact on vision and quality of life, the choice of treatment may vary. In some cases, intra-arterial chemotherapy or intravitreal injections may be

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utilized. Additionally, targeted therapies or immunotherapy may be other options[5]. In this regard, chemotherapy and conventional chemotherapeutic medication such as "Nelarabine" are considered typical therapeutic choices for RB treatment[6]. Nelarabine is a deoxyguanosine analog and a water-soluble prodrug resistant to degradation by purine nucleoside phosphorylase. Recently, this drug has been at the forefront of chemotherapeutic compounds in children with cancer and has had encouraging results[7]. However, contemporary evidence suggests the emergence of drug resistance among cancer cells to anti-cancer medications. Meanwhile, Yoshida et al. have demonstrated that leukemia cells, through the reduction of deoxycytidine kinase expression via epigenetic mechanisms, can induce resistance to nelarabine[8].

With the advancement of nanotechnology, many techniques have been developed to improve drug delivery and increase the efficacy of anti-cancer medications[9]. Niosomes, The mixture of cholesterol molecules that create microscopic lamellar structures, are biocompatible and non-immunogenic structures and deliver hydrophilic and hydrophobic drugs effectively due to their unique construction[10,11]. Noisome-based drug delivery systems are showing promising potential for treating ocular disorders by enhancing drug shelf life and penetration while increasing drug availability to cells. Compared to multiple nanostructures used for ocular drug delivery formulations, Niosomes demonstrate higher chemical stability[12]. Furthermore, current evidence has presented promising potential for niosomes to reduce drug resistance among cancer cells[13,14]. Additionally, due to their enhanced stability, cost-effectiveness, versatile drug-carrying capacity, and potential for targeted drug delivery, niosomes are emerging as a promising alternative to liposomes and other nanoparticle-based drug delivery systems. Their ability to improve drug bioavailability and reduce toxicity makes them particularly compelling for pharmaceutical applications[1,2]. However, to the authors' knowledge, there is still not much evidence of using niosomal medication to treat RB[15].

According to those mentioned above, the current study aimed to evaluate the effectiveness of a new formulation of nelarabine or niosomal nelarabine (Nio-Nelarobine) on RB cancer cells to develop a practical approach for RB improvement and treatment.

Methods

Preparing and multiplying the Y79 cell line

Considering the relevant pathology (Retinoblastoma) and factors such as availability, the study purchased the Y79 cell line from the "Pasteur Institute of Iran." The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Bio-idea, Iran) containing 10% FBS (GIBCO, America), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (Bio-idea, Iran) at 37°C in a humidified 5% CO2 incubator for proliferation to reach the appropriate density. Every three days, the medium of the cells was changed. The current research was approved by the ethics committee of Isfahan University of Medical Sciences. This work has been reported in line with the Animals in Research: Reporting In Vivo Experiments (ARRIVE) criteria[16].

Preparation of niosomes-coated nelarabine nanoparticles

Niosomes were created using the thin film hydration method. Nelarabine (SML1736-10 MG, Sigma, UK) was used at a concentration of 20 µM and in a ratio of 1:20 with surfactants (Tween 80 and Tween 40, Span 80, Span 60, from Merck, Germany), and cholesterol (Sigma-Aldrich, Chol, USA) in a chloroform-methanol mixture (1:2 v/v). The mixture was placed in a rotary vacuum evaporator (Schwabach, Heidolph, Germany) to form a thin lipid film. The organic solvents were removed under reduced pressure at 65°C for 40 minutes. Afterward, the dry, thin film was hydrated with phosphate buffer saline (PBS, pH 7.4) at 65 °C for 1 hour at 10 RCF(g). The prepared suspensions were sonicated for 15 minutes. The niosomal dispersions were left for 30 minutes at room temperature to form vesicles[17].

Measuring nanoparticle size

The average size of nanoparticles was measured using dynamic light scattering (DLS) in a Zetasizer (SZ100, Horiba, Japan) with a 630 nm light beam.

Evaluating nanoparticle structure

The structure of the fabricated nanoparticles was observed and photographed using a field emission scanning electron microscope (Field Emission Scanning Electron Microscopy (FE-SEM), MIRA III, TESCAN, Czech Republic).

Determining the IC50 dosage of niosomal nelarabine

The cellular specimens were initially distributed at a concentration of 5×10^3 cells/well within 96-well plates and incubated for a period of 72 hours. The cells were then stratified into distinct cohorts, including an untreated group (cells lacking niosomal nelarabine) and cohorts subjected to concentrations of 0.2, 0.5, 1, and 2 µM of niosomal nelarabine. The IC50 threshold was established through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) methodology, wherein 10 µl of MTT stock solution (3-[4, 5-dimethylthiazol-2-y1]-2, 5-diphenyltetrazolium bromide; Sigma-Aldrich) was supplemented with 90 µl of the culture medium DMEM (Bio-idea, Iran): F12. Subsequently, the plates were maintained at 37°C with 5% CO_2 for a duration of 3–4 hours. Following this incubation period, the cellular supernatant was aspirated, and 400 µl of dimethyl sulfoxide (DMSO) was introduced into each well to dissolve the formazan crystals formed. After a 15-minute incubation, the plates were agitated, and the optical density was gauged at a wavelength of 540 nm using a spectrophotometer reader (Hyperion MRP, Germany). Each experimental procedure was conducted in triplicate for every group to ensure reliability and consistency.

Cytotoxicity effects of niosomal nanoparticles containing nelarabine

The MTT assay evaluated the cytotoxicity effects of nelarabine loaded onto niosomal nanoparticles. This experiment was conducted for four groups, including untreated(cells without niosomal nelarabine), treated with 1.114 µM of niosomal nelarabine[18], 2 µM of doxorubicin(dox), and niosome (without drug).

Randomization

To minimize bias and ensure the reliability of the results, randomization was applied to allocate cell culture samples into different treatment groups. The cell samples were randomly divided

into four groups using a computerized random number generator, which assigned wells in the 96-well plates to these groups. This process was designed to prevent any bias from influencing group assignments. Furthermore, the identity of the treatment applied to each group was blinded by the researcher analyzing the data to further reduce potential bias. All experimental procedures were conducted in triplicate to ensure consistency and reliability of the results.

Annexin V/PI

Flow cytometric analysis was performed to evaluate apoptosis induction. Annexin V-FITC Apoptosis kit (Invitrogen, USA) was used to assess the effect of niosomal nelarabine applied at 1.114 µM concentrations compared to 2 µM doxorubicin and untreated groups on Apoptosis of Y79 cells cultured in 96 well plates at 10^5 cells per well. Following 72 hours of incubation of Y79 cells under the above conditions, the cells were washed twice with cold PBS, harvested in 500 µL binding buffer, and then incubated with 5 µL Annexin V and PI for 15 minutes at room temperature in a dark The colorless sample was used as a control. Finally, the apoptotic state of Y79 cells was determined by (the BD FACSCalibur machine, Dickinson and Company, Belgium). Graphs were drawn with flowjo software, and statistical analysis with prism9 software.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted from the cells using TRIzol reagent (Kiazist, Iran), and the quantity was checked with nanodrop. Then, the RNA was reverse transcribed for cDNA synthesis using RevertAid first strand cDNA synthesis kit (Parstous, Iran) with oligo dT primer. Real-time was performed with gene-specific primers and the SYBR Green/ROX qPCR Master Mix 2X (Addbio, Korea) and the steponeplus™ Real-time qPCR detection system (Applied Biosystems, USA). To compare the expression levels of the gene of interest, it was normalized using the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A melting curve was generated to determine the melting temperature of the specific reinforcement. These experiments were performed in triplicate and independently repeated at least three times. The relative expression level of each target gene was compared between the treatment group and the reference group and calculated using formula $2^{-\Delta\Delta CT}$[19]. Table 1 shows the sequences of forward and reverse primers.

Table 1

The list of primer sequences (forward, reverse) used in RT-PCR analysis

Gene	Primer
GAPDH	F: AGGTGAAGGTCGGAGTCAAC
	R: CCTGGAAGATGGTGATGGGAT
PTEN	F: TCCTCAGTTTGTGGTCTGCC
	R: AGGTTTCCTCTGGTCCTGGT
FOXO1	F: TCAGAGCCCCCATTTGTTCA
	R: CCCTGGACTTCACTGTTCTCA
AKT	F: CAGCGGGGTAGGGAAGAAAA
	R: TGACAGAGTGAGGGGACACA

Statistical analysis

The statistical analysis was done with Prism 9 software using One-way ANOVA with LSD and Tukey's *post hoc* test. All experiments were repeated three times, and the average data difference was calculated as a significant difference $P \leq 0.05$.

Results

Nanoparticle characterization

Due to the importance of the size of nanoparticle drugs in their performance, the DLS technique was used to evaluate this quantity. According to the results, the estimated mean particle size (D50) was acquired at 167 (SD = 0.2) nm (Fig. 1).

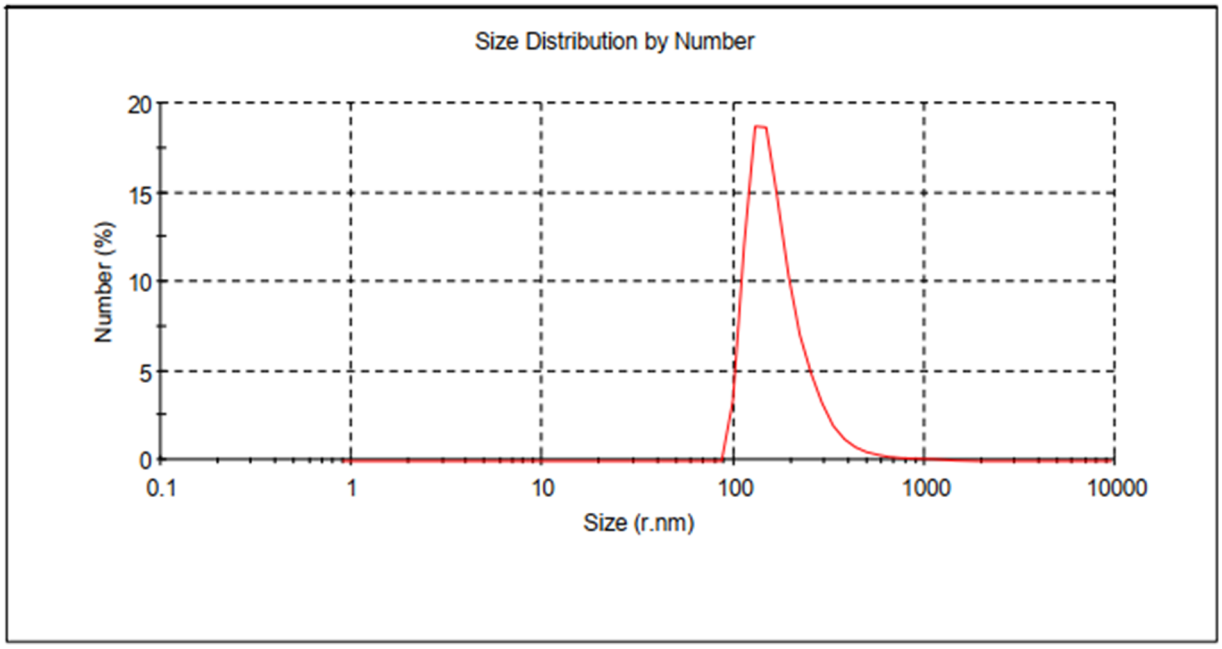

Figure 1. The DLS data is for determining the average particle size of drug-loaded nanoparticles.

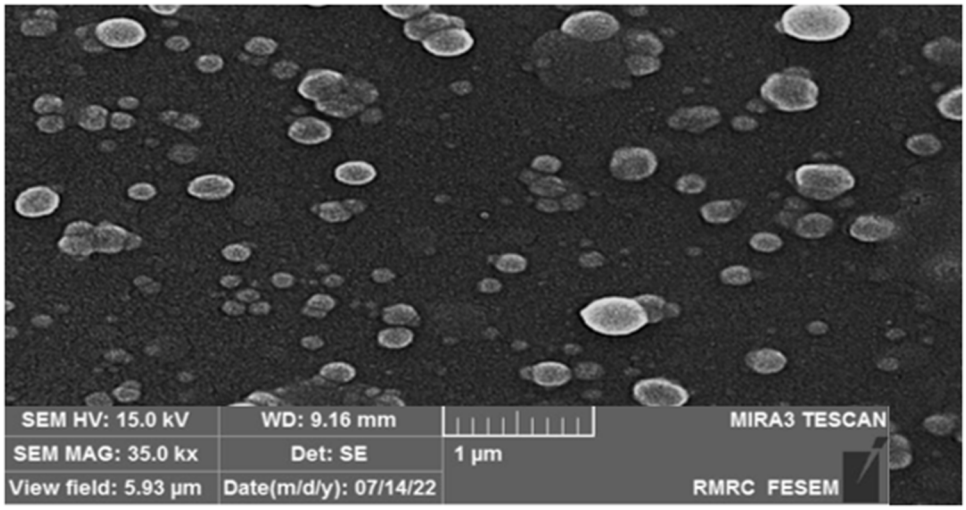

Figure 2. The FE-SEM images of nelarabine-loaded niosomal nanoparticles.

Evaluating nanoparticle structure

As shown in Figure 2, the FE-SEM image revealed that the Nio-Nelarobine had an aspherical shape with a smooth surface. As reported before, the nanoparticles were not monosized, as indicated by the DLS data. DLS measurements indicated that the particle size distribution in most formulations was relatively narrow, suggesting that the niosomes were fairly uniform in size and shape. The analysis of the formulations was conducted at different pH levels to simulate the varying pH values encountered along the carrier's path to its target.

MTT assay

The IC50 assay for inhibitory concentration analysis of niosomal nelarabine on the Y79 cell line was performed at different doses (0.2, 0.5, 1, and 2 µM) compared to the untreated group for 72 hours. As a result, the destruction of half of the cancer cells was considered as IC50 ($P < 0.0001$) (Fig. 3). The cytotoxicity effect of niosomal Nelarabine on Y79 cell lines was determined by MTT assay. The Nio-Nelarobine inhibited cell proliferation in a concentration-dependent manner after 72 hours of treatment with 1.114 µM of Nio-Nelarobine. In addition, the

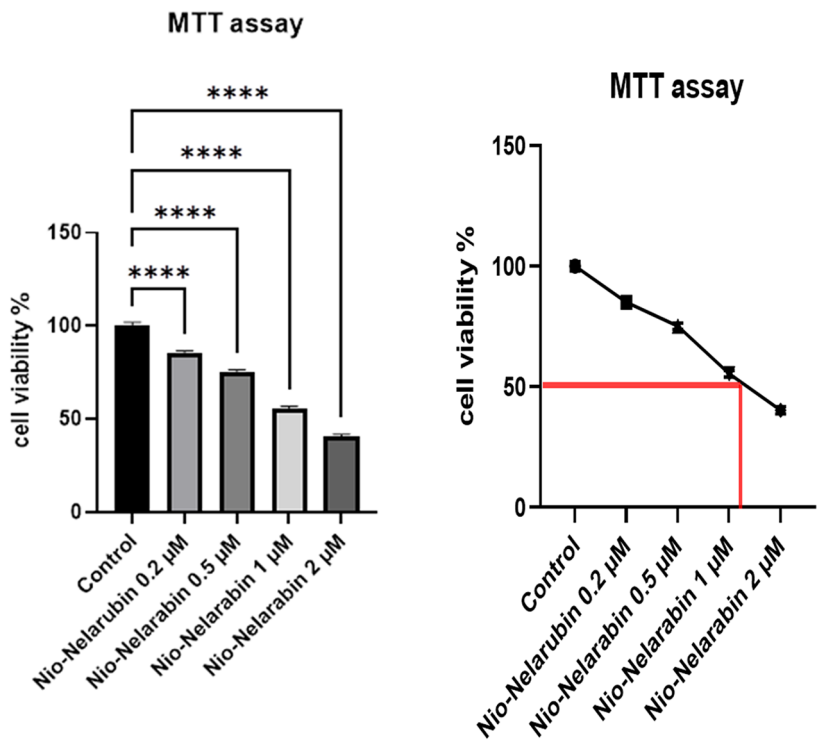

Figure 3. The IC 50 dose of Nio-Nelarobine was determined by MTT assay. Y79 cells were exposed to indicated concentrations of Nio-Nelarabine (0.2, 0.5, 1, and 2 µM) compared to the untreated group for 72 hours (****$P < 0.0001$).

cytotoxicity effect of Nio-Nelarobine (1.114 μM) significantly increased compared to the untreated group(control), niosome (without drug), and doxorubicin, respectively: $P < 0.0001$, $P < 0.0001$, and $P < 0.05$ (Fig. 4).

Apoptosis assessment by flow cytometry (Annexin V–FITC/PI staining)

The effect of Nio-Nelarobine on cell death was assessed via Annexin/PI staining. The percentage of apoptotic cells was determined using the flow cytometry method. According to our finding, Y79 cell line treatment with niosomal nelarabine (1.114 μM) significantly increased apoptosis compared to cells treated with 2 μM doxorubicin ($P < 0.001$) and the control group ($P < 0.0001$) (Fig. 5).

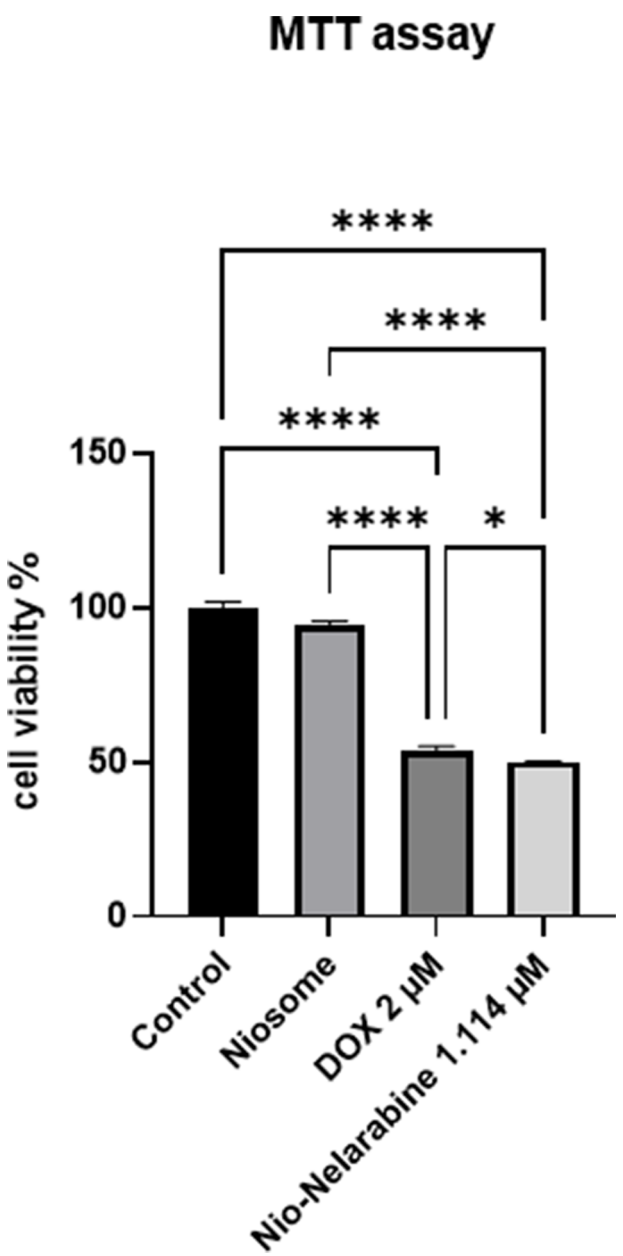

Figure 4. The cytotoxicity effects of Nio-Nelarobine (1.114 μM) and doxorubicin (2 μM) on human retinoblastoma carcinoma cell line Y79 compared to untreated group(control) and niosome (without drug) were evaluated by MTT method. Results were obtained from three independent experiments. Data was represented as mean ± SD (****$P < 0.0001$, *$P < 0.05$).

Real-time PCR

The levels of gene expression, namely AKT, PTEN, and FOXO1, in Y79 cells, post niosomal nelarabine and 2 μM doxorubicin exposure were analyzed through qRT-PCR (Fig. 6). The findings indicated a notable reduction in AKT gene expression in Y79 cells following niosomal nelarabine treatment compared to both the control group ($P < 0.0001$) and the doxorubicin-treated group ($P < 0.0001$). Furthermore, there was a significant elevation in PTEN gene expression in cells subjected to niosomal nelarabine treatment in contrast to the control group ($P < 0.0001$) and the doxorubicin-treated group ($P < 0.001$). Additionally, a marked increase in FOXO1 gene expression was observed in the niosomal nelarabine-treated group relative to the control group ($P < 0.001$) and the doxorubicin-treated group ($P < 0.05$), signifying potential regulatory effects of niosomal nelarabine on these pivotal genes in Y79 cells.

Discussion

RB is regarded as the most prevalent intraocular malignancy among infancy and early childhood, which affects approximately 1 in 20,000 live births[20]. The current results revealed that niosomal nelarabine could enhance the medication's efficacy on cancer cells and induce cancer cell apoptosis. These results can be considered pivotal in paving the way for improving cancer treatment methods.

The current findings indicate that the effective dose of Nio-Nelarobine during 72 hours on Y79 cells was 114.1 μM. Also, The cell survival rate was 94.5% in the Niosome group, 53.7% in the DOX group, and 50% in the Nio-Nelarabine group at the mentioned dose. Additionally, The findings showed a significant decrease in cell survival rates for all groups except for the Niosome group, compared to the control group. Notably, Eletskaya *et al* reported that Nelarabine IC50 was 3 μM for the LS174T cell line, and the variation of the results may be related to variances in cell line and delivery methods[21]. Furthermore, Beesley et al. reported an IC50 of Nelarabine at approximately 2.9 μM for 22 cell lines[22]. Conversely, Annalisa et al. revealed that the IC50 is nearly below 5 μM for the T-ALL cell line[23]. Moreover, by comparing the obtained results, niosome encapsulation of nelarabine could augment the drug efficacy, reducing the dosage. In this regard, Amoabediny et al. demonstrated that niosome, lipid-based vesicles, can encapsulate both hydrophobic and hydrophilic drugs[24]. Similarly, Priya et al. showed that niosomes can facilitate cellular drug absorption owing to their lipid-based nature[25]. This process could enhance the interaction of Nelarabine with Y79 cells, potentially increasing its cellular toxicity.

Moreover, the obtained evidence indicates a significant reduction of AKT expression in the Nio-Nelarobine group compared to other groups. The protein kinase B, also known as AKT, is a serine/threonine-specific protein kinase that plays a crucial role in several cellular processes, such as glucose metabolism, Apoptosis, cell growth, and transcriptional regulation[26]. AKT

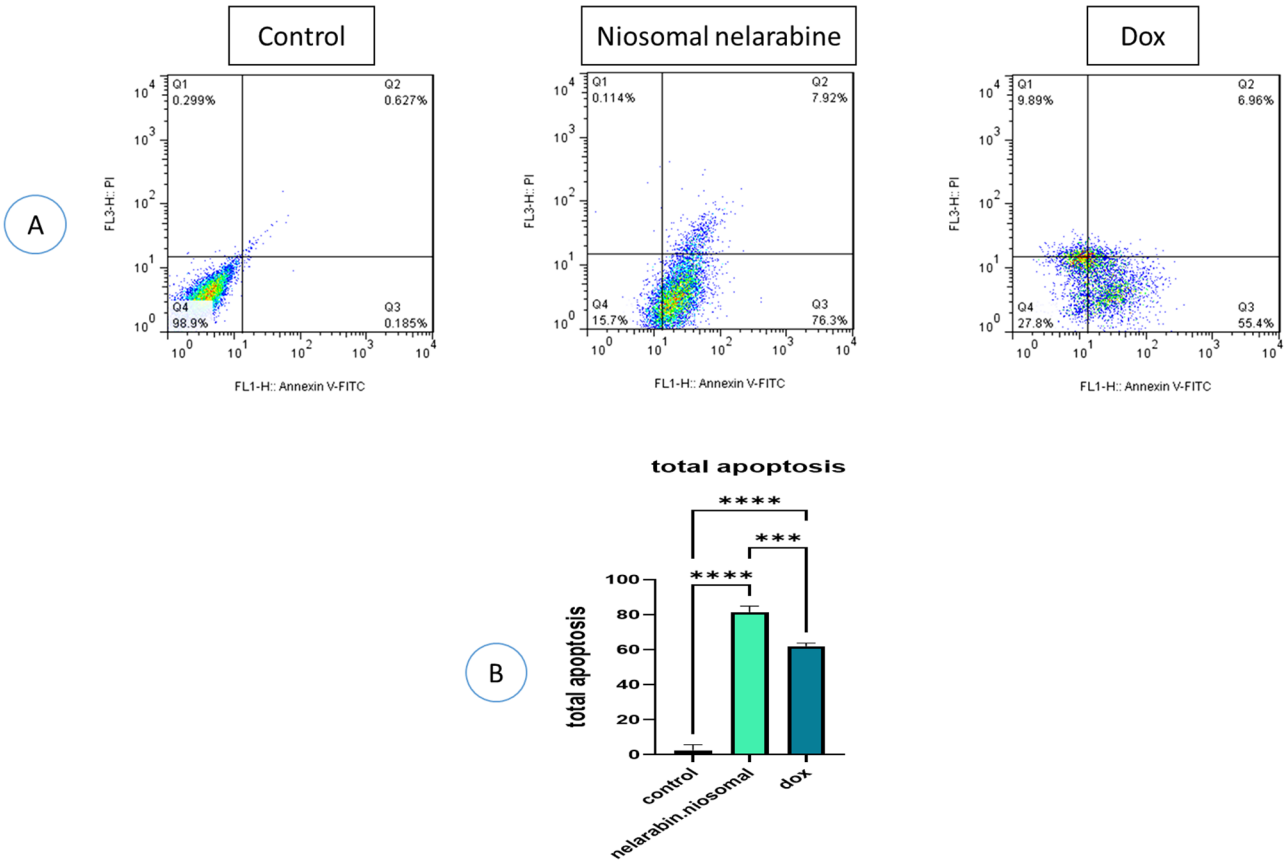

Figure 5. Apoptotic effects of Nio-Nelarobine (1.114 μM) and doxorubicin (2 μM) on Y79 cells. Y79 cells were cultured in 12-well plates and treated in three groups: control or untreated, doxorubicin (2μM), and Nio-Nelarobine (1.114 μM) for 72 hours. Stained apoptotic cells were detected via flow cytometry. All these experiments were repeated at least three times. (A) Dot plots of Y79 cells after culture of these cells with doxorubicin and Nio-Nelarobine. The percentage of apoptotic cells was measured by flow cytometry and analyzed by Cell Quest software. (B) Graphical representation of the percentage of total apoptotic cells in cells exposed to the Nio-Nelarobine compared to doxorubicin and control group. ***$P < 0.001$ indicates statistically significant differences between niosomal nelarabine treatment, 2 μM doxorubicin treatment, and the control group using a one-way analysis of variance (ANOVA).

is activated in many human cancers, primarily due to the loss of the tumor suppressor protein PTEN. Inappropriate activation of AKT has oncogenic properties and is observed in various human cancers[27]. Lonetti et al. have demonstrated that nelarabine treatment with a dose of 5.5 μM increased AKT expression in T-ALL cell lines, which contradicts the present study's findings[18]. Additionally, Rindiarti et al. demonstrated that increased AKT expression may be associated with drug resistance to nelarabine in cancer cells, and combining nelarabine with other therapeutic methods can potentially reduce drug resistance[28]. Various studies have also shown that niosome encapsulation of different anti-cancer medications enhances their effectiveness and ultimately reduces the expression of mediators such as AKT[13,14,29].

The PTEN gene, positioned on chromosome 23q10 and comprising 11 exons, functions as a tumor suppressor. Its role involves regulating cell division by curbing unbridled cell growth and proliferation. Since its discovery in 1997, PTEN has been recognized as a crucial biological marker in cancer. The loss or alteration of PTEN function has been pinpointed across a diverse range of neoplasms, signifying a genetic event linked to tumorigenesis and tumor advancement[29]. In the present investigation, the Nio-Nelarobine-treated group showed a significant increase in PTEN expression compared to other groups. Interestingly, our findings differ from those of Sakhdari et al., who observed that nelarabine administration doesn't increase PTEN expression in T-ALL cells[30]. However, a number of studies have also indicated the indirect enhancing effect of nelarabine on PTEN gene expression through AKT gene reduction in activity[31,32].

Furthermore, the findings indicate a significant upregulation of FOXO1 expression in both treatment groups compared to the control group. Also, the Nio-Nelarobine treated group showed a notably higher FOXO1 gene expression than the doxorubicin group. FOXO1 plays a pivotal role in various processes, including apoptosis, autophagy, antioxidant enzymes, cell cycle regulation, and modulation of metabolic and immune systems[33]. Additionally, it serves as a crucial negative regulatory transcription factor in tumors, contributing to the reduction in survival, growth, and metastasis of cancer cells[34]. The treatments, niosomal nelarabine and doxorubicin, may induce this response to restrict the survival and growth of cancer cells. Additionally, the variation in treatment results can be attributed to these two drugs' distinct mechanisms of action and molecular interactions. In this context, niosomal nelarabine may activate FOXO1 more prominently due to its unique formulation, cellular absorption,

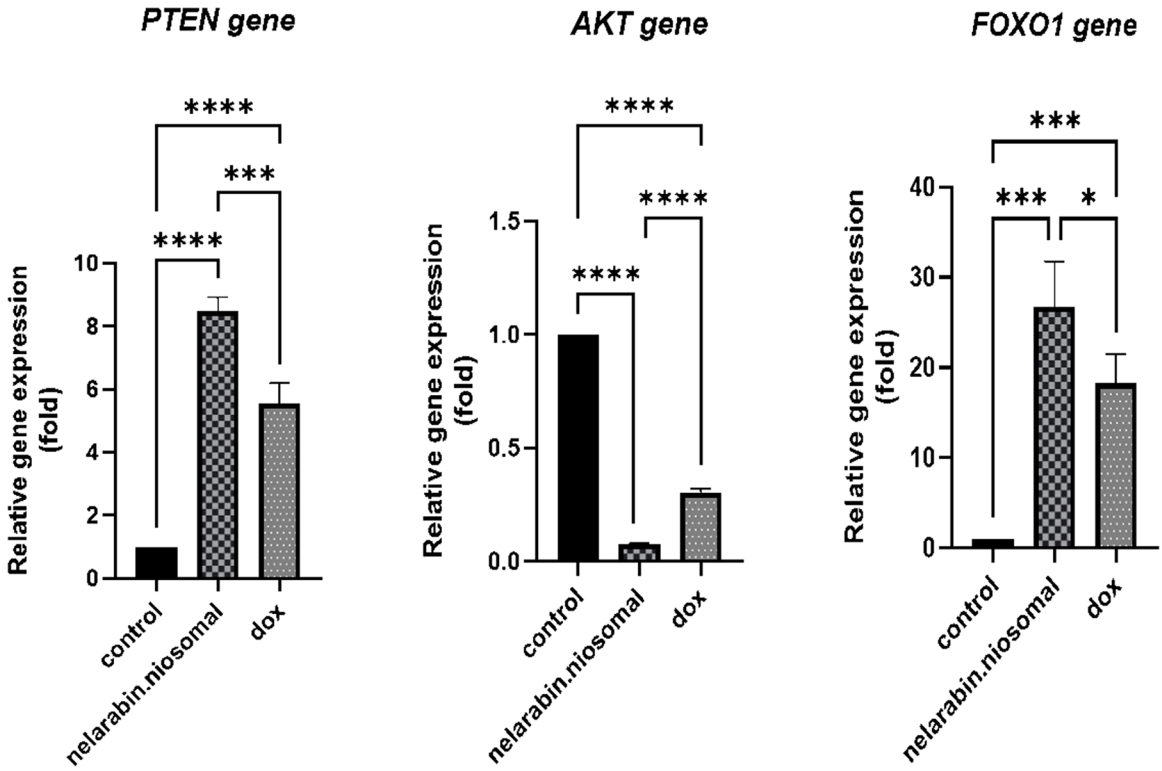

Figure 6. Nio-Nelarobine (1.114 μM) and doxorubicin (2 μM) affect gene expression in the Y79 cell line. Nio-Nelarobine treatment significantly increased the gene expression level of (a) *PTEN* and (b) *FOXO1* in Y79 cells and decreased the gene expression of (c) *AKT* detected by qRT-PCR analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ indicate statistically significant differences between Nio-Nelarobine treatment, doxorubicin treatment, and control group using a one-way analysis of variance (ANOVA).

or the pathways it influences. For example, Hussein and colleagues have suggested that niosomal loaded with melittin can activate FOXO1 more effectively than the free form of this drug, potentially expediting apoptosis[35].

Limitations and strengths

This study is the first to explore the anti-cancer effects of niosomal nelarabine. However, there are some limitations that must be noted. First, this study did not compare the present combination with other nanopharmaceutical approaches. Additionally, this research was conducted *in vitro*, which may have produced some unrealistic effects. Also, only three genes were examined in this study, while more genes involved in key processes such as apoptosis could have been studied. The manuscript, focusing on the use of Niosomes to enhance the efficacy of the drug nelarabine in the treatment of retinoblastoma (RB), has several notable strengths. The innovation in utilizing Niosomes as a drug delivery system demonstrates significant results in increasing cell death (apoptosis) and reducing cancer cell proliferation. Additionally, comprehensive genetic analyses confirm the reduction of AKT gene expression and the upregulation of growth-inhibiting genes such as PTEN and FOXO1, highlighting the anti-cancer mechanisms of this therapeutic approach.

Implications for clinical practice

In light of these findings, developing more effective anti-cancer drugs is a significant concept. On the other hand, the obtained results, particularly the gene expression alteration, pave the way for the targeted development of gene therapy medications with enhanced precision in cancer treatment. This avenue of research holds the potential to revolutionize cancer therapeutics, offering more targeted and efficient treatment strategies for improved patient outcomes.

Recommendations for future research

Future studies should focus on several key areas. First, investigating the underlying molecular mechanisms of niosome drug delivery is essential. This includes examining a wide range of effects on various apoptotic pathways and epigenetic modifications. Second, it is crucial to evaluate the side effects of this therapeutic method on healthy cells, as the cross-interactions of different pathways in this drug delivery method might harm healthy cells while inducing cancer cell death. In the subsequent phases, based on the data obtained, it would be appropriate to conduct animal studies and eventually human trials. Additionally, combining this drug delivery method with herbal medicines, such as tea, could be an effective approach. Finally, it is important to note that this therapeutic method could potentially impact other cancer cell lines besides retinoblastoma, making it an exciting topic for future research.

Conclusion

The current examination showed that nio-Nelarabine could be a promising option for the treatment of retinoblastoma (RB). This nanoparticle formulation demonstrated significant

anticancer effects in the experiments, including increased cancer cell death (apoptosis) and a dose-dependent reduction in cell proliferation. Additionally, genetic analyses revealed that Nio-Nelarabine reduced the activity of the AKT gene, which is associated with cancer cell growth while increasing the expression of growth-inhibiting genes (PTEN and FOXO1). These findings suggest that the use of nanoparticles can enhance drug absorption and improve its effectiveness compared to conventional formulations.

Ethical approval

The current research was approved by the ethics committee of Isfahan University of Medical Sciences, Iran.

Consent

Written informed consent was obtained from the patient for publication and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

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Author contribution

Study concept and design, data acquisition, data interpretation, manuscript drafting, revision, approval of the final version: all authors.

Conflicts of interest disclosure

All the authors declare to have no conflicts of interest relevant to this study.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

Giti Zarinfard.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reason-able request.

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None

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