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Original Article

Liposomal AZD5363 Displays Antiproliferation Activities and Induces Apoptosis on Y79 Retinoblastoma Cancer Cells

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Abstract

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Keywords
- retinoblastoma
- AZD5363
- liposome
- PTEN
- AKT
- FOXO1

Objectives Retinoblastoma (RB) is an aggressive intraocular cancer that usually develops during infancy and childhood. As an Akt kinase inhibitor, AZD5363 is a novel drug whose encapsulation into liposomes enhances its bioavailability and biomedical potential. In the present study, a liposomal membrane was created around AZD5363 to assess its efficacy on the Y79 cancer cell line.

Materials and Methods AZD5363 nanoparticles were synthesized by the thin film hydration method. Dynamic light scattering (DLS) and field emission scanning electron microscopy (FESEM) techniques were applied to evaluate the particle size, and the morphology of the liposomal AZD5363 (Lipo-AZD5363). The MTT test was used to assess the half maximal inhibitory concentration (IC50) of Lipo-AZD5363, and the cytotoxic effects of Lipo-AZD5363 and doxorubicin (Dox) were investigated on the Y79 cell line. Flow cytometry was used to study apoptotic induction in selected groups. Also, the *PTEN/AKT/FOXO1* gene expression level was measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay.

Results Treatment with Lipo-AZD5363 inhibited the proliferation of Y79 RB cancer cell line in a dose-dependent manner. Lipo-AZD5363, at a lower concentration, was significantly more cytotoxic than Dox in terms of enhanced cell death ($p < 0.05$). Furthermore, flow cytometry showed that Lipo-AZD5363 and Dox induce apoptosis in these cells. However, the number of apoptotic cells in the Lipo-AZD5363 group was clearly higher than that in the Dox group ($p < 0.001$). Real-time PCR analysis indicated that Lipo-AZD5363 treatment resulted in an increase in *PTEN* and *FOXO1* gene expression and a decrease in *AKT* gene expression. Our study revealed that all results

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were statistically more significant in the Lipo-AZD5363 group than in the Dox group ($p < 0.01$, <0.01, and <0.001, respectively).
Conclusion Lipo-AZD5363 inhibits proliferation and promotes apoptosis of RB cells by inhibiting the PI3K/AKT signaling pathway. Thus, Lipo-AZD5363 may be a promising candidate for cancer therapy. However, more experimental evidence is needed for its use in the pharmacological treatment of RB.

Introduction

Retinoblastoma (RB) accounts for nearly 4% of all pediatric malignancies and is the most common primary intraocular cancer in childhood. Mutations in the *RB1* gene play an essential role in RB and lead to dysfunction or absence of the RB protein. *PTEN* is a tumor suppressor gene that regulates the cell cycle. Mutation or deletion of this gene often occurs and inactivates or decreases PTEN function.[1] In the absence of growth factors, the FOXO1 proteins translocate to the nucleus when PTEN is active and causes cell cycle arrest, resistance to stress, and apoptosis.[2]

Capivasertib (AZD5363) is a novel drug. It is a selective inhibitor of Akt kinase that suppresses tumor cell proliferation and phosphorylation of AKT substrates in various cell lines.[3]

In this study, the effect of the liposomal formulation AZD5363 on the RB cancer cell Y79 via the PTEN/AKT/FOXO1 signaling pathway was assessed.

Materials and Methods

Materials

Commonly used reagents and consumables include the following: Y79 cell line (Pasteur Institute of Iran), AZD5363 (CAS No. 1.800.364.9897, Cayman Chemical Co, United States), Dulbecco's modified Eagle's high glucose medium (DMEM; Bio-idea, Iran), fetal bovine serum (FBS; GIBCO, United States), penicillin/streptomycin (Bio-idea, Iran), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; Carl Roth, German), Trypsin (Sigma, German), EDTA (Merck, German), TRIzol reagent (Kiazist, Iran), RNase-free water (Thermo fisher Scientific, United States), Easy cDNA Synthesis Kit (Parstous, Iran), SYBR Green/ROX qPCR Master Mix 2X (addbio, Korea), Annexin V-FITC Apoptosis kit (Invitrogen, United States), Dox: Lot No, 8I233L8, Cell Pharma GmbH, Germany.

Cell Culture

Cells were cultured at 37°C in a humidified 5% CO_2 incubator using the DMEM medium that contained 10% FBS and 1% penicillin/streptomycin for their growth. When the cell density reached 80 to 90% confluence, the cell culture medium was replaced. Isfahan University of Medical Sciences (IR.MUI.MED.REC.1401.171) granted approval for the study.

Preparation of Lipid-Coated AZD5363 Nanoparticles

The thin film hydration method was employed for liposome preparation, which consisted of cholesterol, Tween 80, and phospholipids. Soybean phosphatidylcholine (SPC), egg yolk phosphatidylcholine (EPC), and hydrogenated soy phosphatidylcholine (HSPC) were selected as lipids to prepare AZD5363 liposomes. Cholesterol was used to increase membrane rigidity and reduce drug leakage from liposomes.[4] In addition, Tween 80 was applied to enhance liposome encapsulation efficiency. First, phospholipids and cholesterol were dissolved in chloroform, then the mixture was dried by a rotary evaporator (ChongYe RE 3000) at 50°C until a thin film appeared. After that, the film was hydrated using phosphate buffer saline (PBS, pH 6.5) containing 1% Tween 80 and AZD5363 with a concentration of 10 µM/L at 60°C for 30 minutes. The drug-to-lipid ratio was 1:25. The size of liposomes was reduced by a probe sonicator (Noise Isolating Tamber, JY92-IN, Ningbo, China) for 3 minutes, at 80 W.

Particle Size Measurement and Morphology Investigation

Liposomal formulation particle size was measured by dynamic light scattering (DLS; Horiba, Japan). All measurements were performed at room temperature. Lipo-AZD5363 morphology was observed and photographed using a field emission scanning electron microscopy (FESEM; TESCAN, Czech Republic).

Determining the IC50 Dosage of Lipo-AZD5363

The half maximal inhibitory concentration (IC50) dosage was determined by the MTT method. The 5×10^3 cells were seeded into a 96-well plate at 37°C with 5% CO_2. Y79 cells were divided into groups, including untreated (control) and those treated with 0.37, 0.75, 1.5, and 3 µM of Lipo-AZD5363. After 72 hours, the MTT stock solution (5 mg/mL MTT in PBS) with DMEM/F12 was added to each well at a 1:10 ratio and the plates were incubated at 37°C for 3 to 4 hours. After applying this solution, the environment turned blue due to formazan production. The supernatant of the cells was discarded and 200 µL of dimethyl sulfoxide (DMSO) was added to extract MTT formazan. All experiments were done in three replicates for each group. The absorbance was detected by a microplate reader (Hyperion MRP 4+, Germany) at a 540-nm wavelength.

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Cell Cytotoxicity Assay

The MTT assay evaluated the cytotoxicity of Y79 cells into four groups: untreated (control), and those treated with 0.9 µM of Lipo-AZD5363, 2-µM doxorubicin (Dox), and liposome (without drug).

Detection of Apoptosis Using Flow Cytometry

Cell apoptosis induction was assessed using propidium iodide (PI)/Annexin and analyzed through flow cytometry. The cells were divided into untreated (control) and treated with 0.9 µM of Lipo-AZD5363 and 2-µM Dox. The Y79 cells were treated for 72 hours and washed twice with PBS. In total, 1×10^5 cells were poured into a microtube and brought to a volume of 500 µL with the binding buffer (1x) included in the kit. One sample was used as a control without color to set up the device. The microtube containing the sample was mixed with 5 µL of Annexin V-FITC. Then it was incubated for 15 minutes at room temperature and in the dark condition. Also, 5 µL of PI was added to the sample and the reading was done using a flow cytometry device (BD FACSCalibur, Dickinson and Company, Belgium). Data were obtained and analyzed using Flowjo software. These experiments were done with three replicates in each group.

Quantitative Real-Time Polymerase Chain Reaction Assay

Extraction of total RNA was done according to the Trizol manufacturer's protocol. The RNA was extracted from Y79 cells according to the instructions. The isolated RNA was dissolved in RNase-free water. The purity and concentration of the RNA were assessed by measuring the absorbance at 260 to 280 nm with a nanodroplet. Total RNA was consumed to synthesize double-stranded cDNA using the Easy cDNA Synthesis Kit and an oligo-dT primer. The primers were designed for all assessed genes (Sinaclon, Iran). The primer sequences used in this study were according to following: *AKT* (Forward: CAGCGGGGTAGGGAAGAAAA, Reverse: TGACAGAGTGAGGGGACACA), *PTEN* (Forward: TCCTCAGTTTGTGGTCTGCC, Reverse: AGGTTTCCTCTGGTCCTGGT), *FOXO1* (Forward: TCAGAGCCCCCATTTGTTCA, Reverse: CCCTGGACTTCACTGTTCTCA), *GAPDH* (Forward: AGGTGAAGGTCGGAGTCAAC, Reverse: CCTGGAAGATGGTGATGGGAT).

RT-PCR was performed using gene-specific primers and Maxima SYBR Green/ROX qPCR Master Mix 2X and the StepOnePlus RT-PCR detection system (ABI StepOne, United States). The melting curve analysis was utilized to determine the melting temperature of particular amplification products and the primer. The experiments were conducted in triplicate replicates, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was employed as a maintenance gene. Using the $2^{-\Delta\Delta CT}$ method, the expression level of each target gene was determined.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by the Tukey test was applied to determine the statistical significance of data. A *p*-value less than 0.05 was calculated as a statistically significant difference. The data were statistically analyzed with GraphPad Prism 9 software.

Results

Nanoparticle size was characterized using DLS. The average particle size was 224 ± 3.2 nm.

The MTT data revealed that the IC50 of Lipo-AZD5363 was 0.9 µM, significantly inhibited the survival of RB the Y79 cells in comparison to the control group, and the inhibitory effect on cell proliferation was dose dependent ($p < 0.0001$). Moreover, the cytotoxicity effect of Lipo-AZD5363(0.9 µM) significantly increased compared with the untreated group, liposome (without drug), and Dox, respectively ($p < 0.0001$, <0.0001, and <0.05; ►Fig. 1).

Also, our data indicate that the apoptosis rate on the Y79 cell line treated with Lipo-AZD5363 (0.9 µM) significantly increased compared with cells treated with 2-µM Dox ($p < 0.001$) and the control group ($p < 0.0001$; ►Fig. 2).

The results of RT-PCR revealed that Lipo-AZD5363 (0.9 µM) significantly decreased the expression levels of *AKT* compared with the control group ($p < 0.0001$) and the Dox-treated group ($p < 0.01$). Also, the expression of the *PTEN* gene in the group treated with Lipo-AZD5363 increased significantly compared with the control group ($p < 0.0001$) and the group treated with Dox ($p < 0.01$). An increase in the *FOXO1* gene expression was observed in the group treated with Lipo-AZD5363 compared with the control group ($p < 0.0001$) and the group treated with Dox ($p < 0.001$; ►Fig. 3).

Discussion

AZD5363, a strong pan-Akt kinase inhibitor, inhibits human tumor xenografts monotherapeutically.[5] According to recent

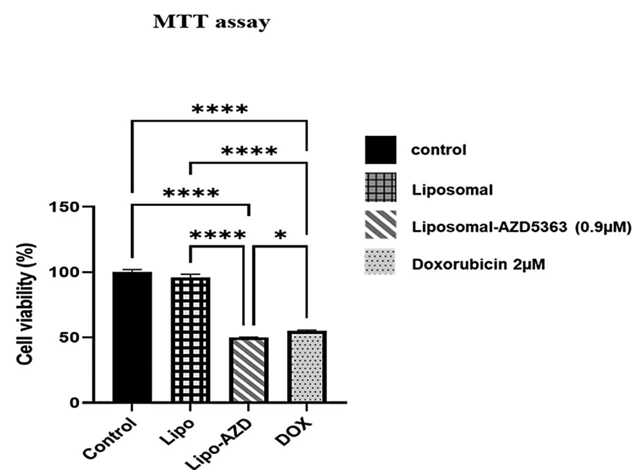

Fig. 1 The cytotoxicity effects of Lipo-AZD5363 (0.9 µM) and doxorubicin (2 µM) on human retinoblastoma carcinoma cell line Y79 compared to untreated group (control) and Liposome (without drug) were evaluated by MTT method. Results were obtained from three independent experiments. Data was represented as mean SD, $p < 0.0001$), ($p < 0.05$).

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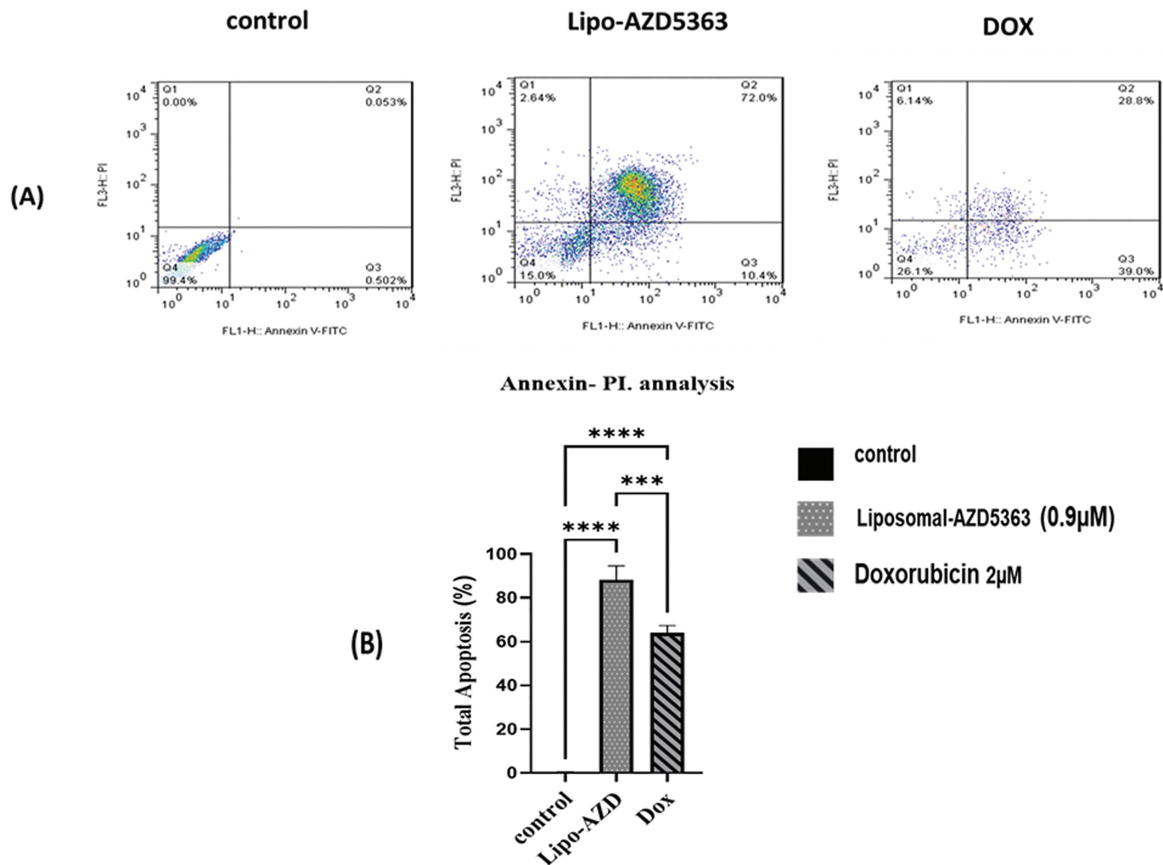

Fig. 2 Apoptotic effects of Lipo-AZD5363 (0.9 μM) and doxorubicin (2 μM) on Y79 cells for 72 hours. Stained apoptotic cells were detected via flow cytometry. All these experiments were repeated at least three times. (A) Dot plots represent the Anexin V/PI positive Y79 cells after culture of these cells with doxorubicin and Lipo-AZD5363. (B)Graphical representation of the percentage of total apoptotic cells in cells exposed to the Lipo-AZD5363 (0.9 μM) 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).

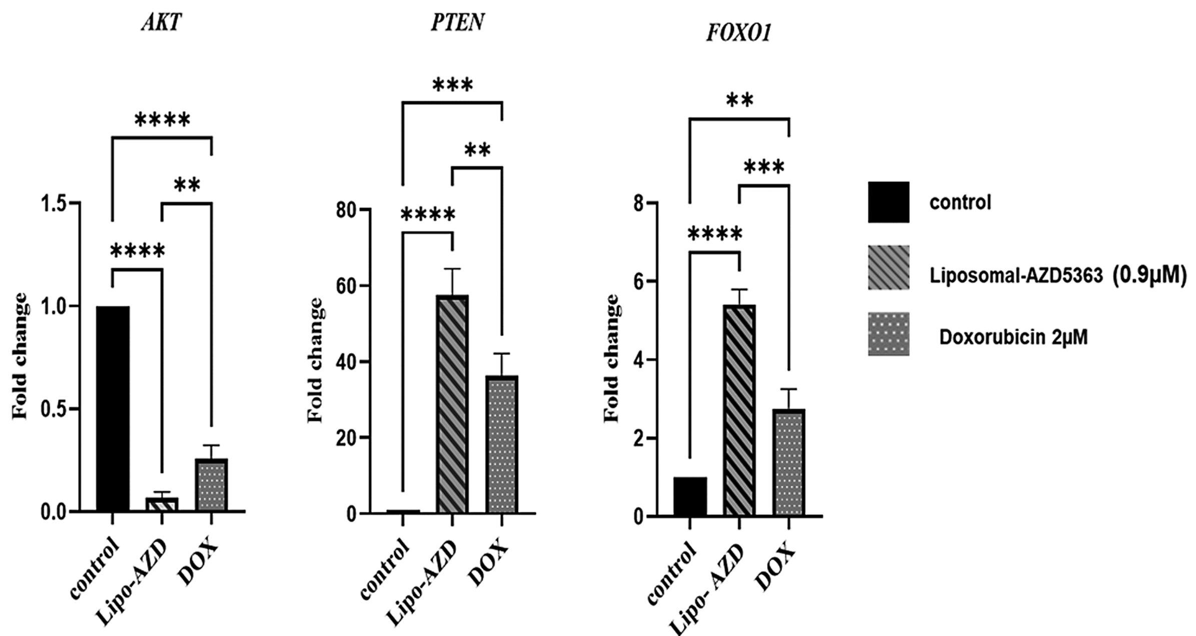

Fig. 3 Comparative analysis of AKT, PTEN, and FOXO1 mRNA expression on Y79 cells examined by reverse transcription polymerase chain reaction (RT-PCR). The expression level of the AKT gene was significantly downregulated in Lipo-AZD5363 (0.9 μM) as compared with Dox (mean standard error of the mean [SEM], $p < 0.01$). The expression levels of the PTEN and FOXO1 genes were significantly upregulated in Lipo-AZD5363 (0.9 μM) as compared with Dox (mean SEM, $p < 0.01$ and $p < 0.001$).

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studies, liposomes can control drug release and reduce the toxicity in normal tissues.[6,7]

We found that Lipo-AZD5363 at a lower concentration had significantly more cytotoxic effects than Dox (2 µM). Furthermore, our findings showed a significant decrease in cell survival rates for all groups, except the liposome group, compared with the control group. Zhang et al demonstrated that the AZD5363 IC50 concentration was lower when prolonging the cell exposure.[8] Nevertheless, Lamoureux et al revealed that AZD5363 decreases cell proliferation after 2 days at 10 µmol/L. Differences in results may be due to variability in cell lines and delivery methods. In the current experiment, the apoptosis percentage of Y79 cells after treatment with Lipo-AZD5363 or Dox increased compared with the control group. Also, Lipo-AZD5363 promoted the apoptosis of Y79 cells more than the Dox group. In addition, Lipo- AZD5363 had a lower necrotic effect than Dox. Consistent with our findings, Şanli et al demonstrated that AZD5363 successfully suppresses proliferative, clonogenic, and migratory features of the HEp-2 cells through induction of apoptosis.[9]

Proliferation and apoptosis of cancer cells are controlled by the PI3K/AKT pathway.[10] AZD5363 suppresses proliferation through the inhibition of AKT kinase activity in vitro. In addition, AZD5363 downregulates the phosphorylation of downstream pathway proteins dose dependently.

In the present investigation, the Lipo-AZD5363-treated group showed a significant decrease in *AKT* expression compared with other groups. According to Mejía-Rodríguez et al, the PI3K/AKT and SHH survival pathways are hampered by the combined treatments with AZD5363 + AZD8542 + Curcumin and AZD8542 + Curcumin + Resveratrol.[11] Resistance to various therapies in both preclinical and clinical trials is induced by the negative regulator of the PI3K/AKT pathway, tumor suppressor phosphatase (PTEN), which is either deleted or silenced.[12]

Our findings indicated that the gene expression of *PTEN* and *FOXO1* in the Lipo-AZD5363 group significantly increased after treatment compared with the other groups. Consistent with our findings, Sun et al demonstrated that an increase in the *PTEN* expression significantly inhibits cell proliferation and induces apoptosis of colorectal cancer cells via the PI3K/AKT signaling pathway.[13] Also, we found that in the Lipo-AZD5363 group, compared with other groups, the expression of *FOXO1* as a downstream factor increased and the *AKT* gene that inhibits it decreased significantly.

Fabi et al showed that the combination of AZD5363 with Dox induces FOXO1and promotes apoptosis in ovarian and endometrial cancer cell lines better than the combination of NVP-BEZ235 with cisplatin.[14]

Conclusion

This study demonstrated that Lipo-AZD5363 provides an effective means of inducing apoptosis and inhibiting proliferation in RB through the regulation of the PI3K/AKT signaling pathway. Further studies are required to validate the preventive effects of Lipo-AZD5363 on RB, even though the current study offers promising results for an antitumor drug for RB.

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Conflict of Interest

None declared.

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