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Comparing the Effects of Free and Liposomal Indole Compounds on Bax and Bcl2 Gene Expression Changes in the KG-1 Cell Line

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Abstract

Background: For patients with acute myeloid leukemia (AML), the long-term survival rate is still very low. This study examines the effects on AML cell lines of an indole chemical in its free and liposomal forms.

Material and Method: In this experimental case control study, an AML-originated KG-1 cell line was cultured in RPMI 1640 medium. The cells were treated with the free and liposomal forms of an indole compound (C18H10N2F6O) at different concentrations of 20, 40, 100, 200, and 400 µg/mL after they attained the proper confluence. The cellular metabolic activity was examined by an MTT assay. The expression of BAX and BCL-2 genes was investigated by q-PCR to assess the apoptotic effect of that compound. The analysis was also done between each experimental group and the control group using t-test. $P<0.05$ was assumed significant.

Results: Based on the MTT assay, the lethal effective dose of free indole was found to be 245.1 µg/ml and 164.8 µg/ml in 24 and 48 hours, respectively. The corresponding values for liposomal indole were 47.2 µg/ml and 40.6 µg/ml. Furthermore, treatment with free and liposomal forms of indole resulted in a decline in the expression level of the BCL-2 gene. However, in the case of the liposomal compound, this decrease was only statistically significant after 48 hours of treatment ($P < 0.05$). Furthermore, the expression of BAX gene increased after treatment with both free and liposomal forms of indole, but it significantly increased only after treatment with the liposomal compound ($p < 0.05$).

Conclusion: These results suggest that an indole derivative, especially when liposomal, causes apoptosis in AML cells, hence exhibiting cytotoxic effects. To confirm the potential usefulness of this indole derivative as a therapeutic agent for inhibiting tumor progression in the setting of human malignancies, more studies on physiologically relevant models are necessary.

Keywords: AML, Apoptosis, BAX, BCL2, Indole, Liposome

Introduction

Acute myeloid leukemia (AML) is a cancerous condition that inhibits the myeloid lineage from differentiating and increases the number of immature myeloid progenitor cells in patients' bone marrow. The majority of instances occur due to genetic mutations, despite a small percentage of patients who are afflicted for causes such as prior chemotherapy or exposure to certain chemicals (1). There are different treatment options for AML, including chemotherapy, surgery, and radiation, but chemotherapy is the most common one (2). Unfortunately, individuals who are weak or old may not be able to withstand intense chemotherapy; as a result, they require more efficient treatment plans. Additionally, the effectiveness and response rate of alternative therapy options are very low (3). Heterocyclic compounds are cyclic substances with distinct structural variations that have a range of biological functions (4). Indole derivatives are one of

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these substances with anti-tumor effects. They have a bicyclic structure with a six-membered ring connected to a five-membered pyrrole ring that contains nitrogen (2). Research has shown that the majority of indole derivatives have wide anticancer effects on a range of cancerous cell lines, such as human ovarian adenocarcinoma (SK-OV-3), colon carcinoma (HT-29) and c-Src kinase activity (5). Also, 3-pyranyl indoles are cytotoxic to MCF-7 breast cancer cell lines (6).

Nanotechnology improves the solubility and stability of medications, extends their plasma half-life, reduces their adverse effects, and concentrates their actions on a target location. A significant family of biodegradable nano-carriers called liposomes increases medicine delivery to the tumor site while significantly reducing the corresponding adverse effects. Liposomes, which can be prepared mainly with a variety of phospholipids, are safe and biodegradable structures in terms of biliary (7). The present study assesses the effects of indole-3-carboxaldehyde, an indole derivative, on AML cells (KG-1). The first objective of the study is to examine the potential loading of indole compounds into liposomes. Subsequently, the expression of pro- and anti-apoptotic genes is measured to determine whether the enhanced bioavailability of this formulation leads to increased biological activity.

Materials and Methods

The laboratory of Rafsanjan University of Medical Sciences, located in Rafsanjan, Iran, was the site of this experimental case control study. The research and its tools were authorized by the Research Ethics Committee of Rafsanjan University of Medical Sciences (Code of ethics: IR.RUMS.REC.1396.115), and ethical standards were practiced throughout the study.

Indole compound characterization and the preparation of indole-loaded liposomes

The indole compound 2-(2,2,2-trifluoro-1-((2-(trifluoromethyl)phenyl)imino)ethyl)-1H-indole-3-carbaldehyde was received from the Department of Chemistry at Valiasr University (Rafsanjan, Iran). The information about this compound is given in Table I. Soybean phospholipids with 75% phosphatidyl-choline, 2-distearoyl-sn-glycero-3-phosphoethanol-amine, and sodium salt (DSPE-mPEG-2000) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was also purchased from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals and solvents used in this study were of analytical grades.

SPC, cholesterol and PEG were used in a 70:30:2 molar ratio to create liposome vesicles. To create indole-containing nanoparticles, a thin-film hydration-sonication procedure was used. Using a rotary, hydration was accomplished by adding deionized water at 65°C for 60 minutes. Probe sonication was also done in a 45-minute size reduction operation. A PBS buffer was added to a dialysis bag containing the sample to finish the process of isolating the free medication. At room temperature, each measurement was done in triplicate, and the mean result was recorded.

Encapsulation efficiency

At first, a cellulose membrane dialysis tube (cutoff: 12–14 kDa) was filled with indole-loaded liposomes to separate out un-encapsulated medication. After the liposomal solution was solved with isopropanol (99% purity), the quantity of liposomally encapsulated indole was measured using a UV spectrophotometer (model T80+, PG Instruments, United Kingdom) at 480 nm. To examine the relationship between the concentration of DOX and its absorbance through a sequence of dilutions of a doxorubicin

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mixture in isopropanol, a standard curve of DOX was drawn at 480 nm. The encapsulation efficiencies were determined as follows:

$$Encapsulation\ efficiency\ (\%) = \frac{The\ amount\ of\ indole\ encapsulated\ in\ liposome}{(Total\ amount\ of\ the\ indole\ added)} \times 100$$

In vitro drug release

The diffusion of indole from liposomes was studied through dialysis (MW cutoff = 12 kDa, Sigma, Germany) against PBS with the pH values of 7.4 and 5.4 at 37°C and 42°C for 48 hours, respectively. The dialysis medium was taken at various periods and then quickly substituted with a similar proportion of fresh PBS in order to calculate the released indole. A UV spectrophotometer at 480 nm was used to evaluate the samples. At each time interval, the percentage of the release was determined based on the overall dose of the liposome formula.

Physical characterization of liposomal vesicles

Using the Zeta-Sizer device (DLS, Malvern Zatasizer Nano-ZS, Worcestershire, UK), the surface charges (zeta potential) and the liposomal hydrodynamic diameters (particle size) were measured. The samples were prepared and measured right away after being diluted in deionized water (0.1 mg/ml), which served to detect the scattered light at room temperature at an angle of 90 degrees. All the measurements were performed three times, and the mean values were computed. The average polydispersity index (PDI) of liposomes was also calculated.

Cell culture

The AML-originated KG-1 cell line was obtained from the Pasteur Institute of Iran in Tehran, Iran. The cells were cultivated in an RPMI 1640 medium (Shellmax, Iran) that was further enriched with 10% fetal bovine serum (GibcoBRI, USA) and 2% penicillin/streptomycin (Shellmax,Iran) in an atmosphere of CO2 (5%) and O2 (95%) at 37°C temperature. The indole core-based derivative (C18H10N2F6O) was used to treat the cells once they attained the proper confluence (close to 70%), along with its liposomal forms at the doses of 1000, 500, 250, 125, 62, 31, 15 and 8 g/mL. Moreover, 100 mL of dimethyl sulfoxide (DMSO) from MERK, Germany, was used to make the stoke. 900 µ of the RPMI medium was then added to create the working solution. The cells were grown in six-well plates for treatment (SPL, South Korea). It should be mentioned that different chemical dosages were employed during this procedure.

MTT assay

The MTT test was used to assess cellular metabolic activity. First, the KG-1 cells were seeded in a plate (96-well) for 24 hours. The cells were subsequently subjected to three different series of tests, each involving an equivalent volume of a new media and different quantities of empty liposome, liposomal indole, and free indole:

- Control (fresh medium, 200 µl)
- Empty liposomes (20 µl empty liposome + 180 µl fresh medium)
- Free indole (1000, 500, 250, 125, 62, 31, 15 and 8 g/mL)
- Liposomal indole in various concentrations (1000, 500, 250, 125, 62, 31, 15 and 8 g/mL)

The re-incubation lasted for 24 and 48 hours. Then, 20µl (5 mg/ml) of MTT was applied to each 96-well plate and allowed to be incubated for three hours. The supernatant was then removed, and 180µl of DMSO was added to dissolve the crystals. The EPOCH microplate spectrophotometer (synergy HTX, Bio Tek, USA) was used to measure the absorption at 570 nm. These measurements

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were done to establish IC50 doses (the amounts of active components required to inhibit cell growth by 50%) in all the tests.

Total RNA extraction and cDNA synthesis

Free and liposomal forms of the indole compound were applied to KG-1 cells at IC50 concentrations. Then, based on the kit's guidelines, the Trizol reagent (Invitrogen, USA) was used to extract the cellular total RNA content of the cultivated KG-1 cells. Using a nano-drop spectrophotometer, the absorbance at 260/280 nm was measured to estimate the RNA concentrations. For mRNA quantification, the first-strand complementary synthesis reaction was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) based on the kit's guidelines. The reverse transcription reaction products were used in quantitative polymerase chain reactions (qPCR) to amplify the selected mRNAs. The product of the cDNA synthesis was kept at -20°C until it was needed.

Quantitative PCR and gene expression

Using specified primers, the relative expressions of Bcl-2, Bax were evaluated through quantitative real-time PCR (qRT-PCR). The reference genes for the normalization of candidate genes were GAPDH in a row. The specific primers for Bcl-2, Bax and GAPDH and their expected product sizes are reported in Table II. The PCR was run using SYBR Green qPCR Master Mix 2X (Ampliqon, Denmark) on the StepOne™ real-time PCR system (Life Technologies Co., Taiwan) following the manufacturers' protocols. The initial denaturation step and polymerase activation were carried out for two minutes at 95°C during the PCR thermal cycling conditions. This was followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 30 seconds. To evaluate the specificity of amplification, the reaction was continued with a melt curve step at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.

Statistical analysis

The collected data were statistically analyzed with the Graphpad prism software (version 9). The data analysis was done using the comparative Ct ($2^{-\Delta ct}$) method. All the experiments were performed in duplicate to minimize the sampling error for each individual sample, and the results were all presented as the average values yielded in those three experiments. The statistical analysis was also done between each experimental group and the control group using the t-test, and a p-value of less than 0.05 was considered to be statistically significant.

Results

Encapsulation efficiency

By dissolving liposome solution in isopropanol to break down lipid membranes and allow the entrapped indole to seep into isopropanol, the effectiveness of encapsulating indole into nanoparticles was ascertained indirectly. This was followed by spectrophotometry. It was found that the indole encapsulation efficiency into liposomes was 48.4%.

In vitro thermo-and pH-sensitive indole release assay

The pH level in the blood stream is 7.4, and the pH of 5.4 is the value which nanoparticles encounter in a tumor area. In this regard, hypoxia, a condition in which cancerous cells are deprived of oxygen, causes a reduction of pH inside the cancer site.

The in vitro release of indole from the prepared liposome vesicles was monitored for two days in a PBS buffer with pH 7.4 at 37°C and pH 5.4 at 42°C. As illustrated in Figure 1, the indole compound was released from the beginning to the end with a gentle slope. The release of the indole compound occurred within two days and so slowly that, in a period of 48 hours, the maximum release at 37°C and

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42°C was 38.3% and 46.8%, respectively. It is concluded that the release of indole is significantly higher at lower pH levels, ensuring the correct timing of release inside cancer cells.

Characterization of liposomal indole

The size of the indole-loaded nano-liposome was 56.0 nm, and its PDI was 0.29, which means no aggregation occurred. The zeta potential of nano-liposome indole was also -2.05 ± 0.63.

MTT assay results

According to the findings on the MTT assay, the lethal effective dose of free indole was 245.1 µg/ml in 24 hours and 164.8 µg/ml in 48 hours. Also, the lethal effective dose of the liposomal system containing indole was 47.2 µg/ml in 24 hours and 40.6 µg/ml in 48 hours. This indicates that the toxicity of indole is increased due to encapsulation and that nanoparticles do not contain toxic drugs (Figure 2).

Effect of C18H10N2F6O on the expression of BAX and Bcl-2 genes

As the quantitative RT-PCR data showed, treatment with free and liposomal indole could decrease the expression level of BCL-2 in the AML cell line compared to the control cells. This decrease was observed after 24 and 48 hours of treatment, but it was only statistically significant after 48 hours of treatment with liposomal indole ($P < 0.05$).

However, the findings of the research showed that treatment with both free and liposomal indole would lead to an increase in the expression level of the BAX gene in the AML cell line. This increase was observed after 24 and 48 hours of treatment and was statistically significant following the treatment with liposomal indole (Table III). This suggests that the expressions of both *Bax* and *Bcl-2* genes follow a time-dependent pattern.

Table I: New indole compound features

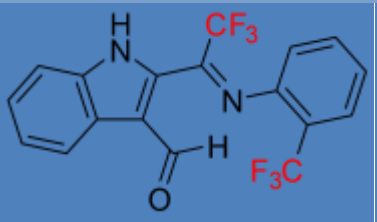
969516	**PubChem ID**		**Two-dimensional structure**
$C_{18}H_{10}N_2F_6O$	Molecular formula		
384.28 g/mol	Molecular weight		
Chemical name			
Trifluoromethylated indole			
2-(2,2,2-trifluoro-1-((2-(trifluoromethyl)phenyl)imino)ethyl)-1H-indole-3-carbaldehyde			**Name IUPAC**
An indole compound with a cream-yellow color and fluorinated compounds are widely used in the pharmaceutical industry due to their unique biological properties.			**Biological source**
It is known for its wide range of anti-rheumatoid, antioxidant, anti-cancer and anti-HIV functions.			**Biological effects**
Melting point	Physical condition	Color	**Physical and chemical properties**
114-115 °C	Solid	Cream-yellow	
Soluble in ethanol, ether, chloroform and dimethyl sulfoxide solvents		Solubility	
Stable at room temperature		Stability and decomposition	

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Table II. List of primers used in this study

Gene Name	Primer sequences (5`-3`)	Product Size (bp)
BAX	F: AGATCATGAAGACAGGGGC R: AGACACTCGCTCAGCTTCTT	**136**
BCL2	F: GCCCTGTGGATGACTGAGTA R: GAAATCAAACAGAGGCCGCA	**117**
GAODH	F: CAAGAGCACAAGAGGAAGAGAGAG R: TCTACATGGCAACTGTGAGGA	**103**

Table III: The Expression of BAX and BCL-2 genes through the treatment with free indole (Base) and liposomal indole (LIPO) each after 24 and 48 hours

Relative expression of genes (2^-Δct)	Control (mean±SD)	Base 24 h (mean±SD)	Base 48 h (mean±SD)	LIPO 24 h (mean±SD)	LIPO 48 h (mean±SD)
BAX	1.32±0.32	2.33±0.11	2.43±0.17	3.81±0.92	3.66±0.56
p-value		0.0527	0.0510	0.0696	0.0362*
Bcl-2	0.05±0.01	0.038±0.009	0.023±0.005	0.021±0.005	0.010±0.002
p-value		0.3213	0.0958	0.0823	0.0450*

The gene expression results were presented as mean ± SD of (2^-Δct), and a statistical analysis was done between each of the groups and the control group using t-test. $P < 0.05$ was determined as the statistically significant level.

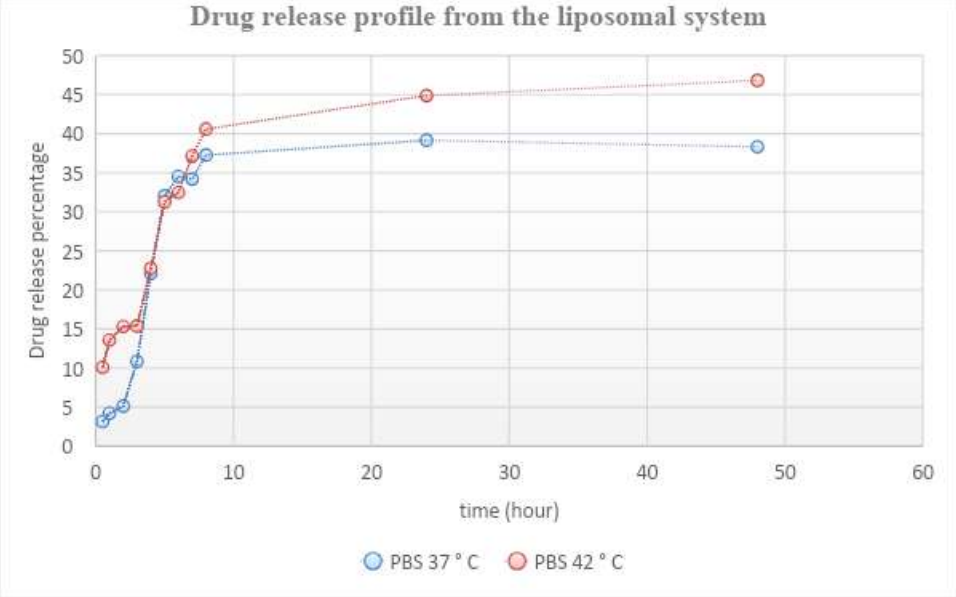

Figure 1. In vitro kinetic release of drug at various pH values and temperatures

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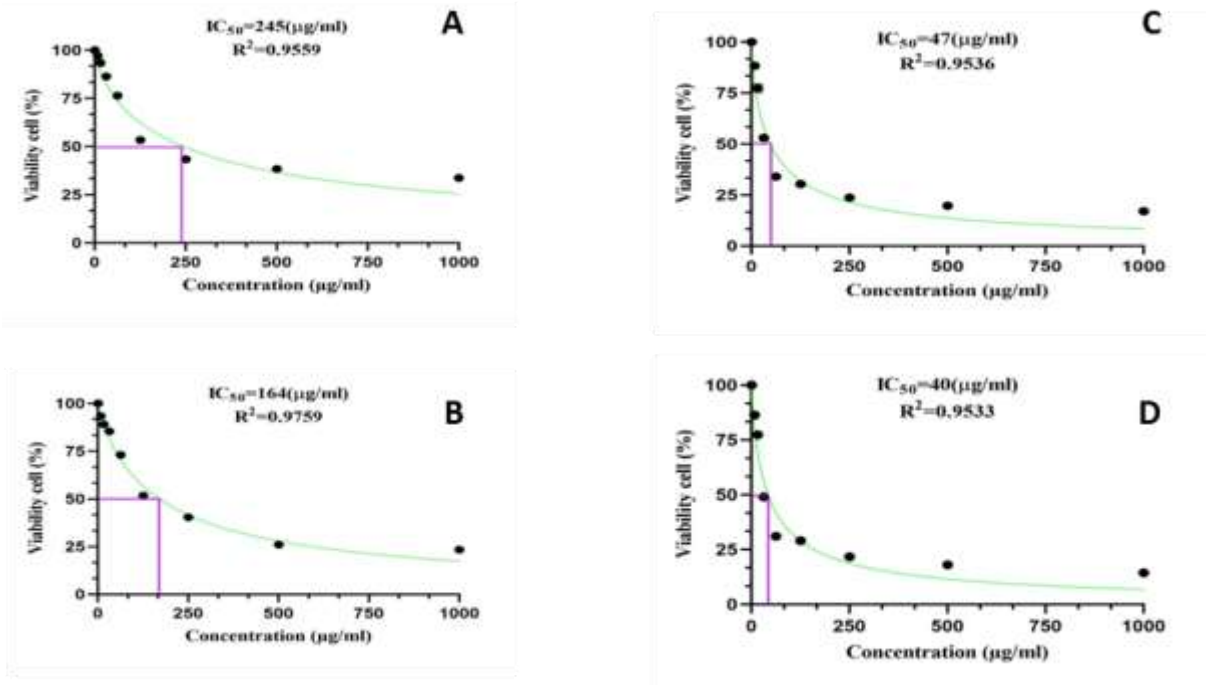

Figure 2. Results of MTT assay: a) Lethal effective dose of free indole in 24 h, b) Lethal effective dose of free indole in 48 h, c) Lethal effective dose of liposomal indole in 24 h, d) Lethal effective dose of liposomal indole in 48 h

Discussion

A combination of daunorubicin (DNR) and cytarabine (Ara-C) is a standard regimen for AML therapy. While the rate of complete remission has increased with the introduction of targeted treatments used in conjunction with chemotherapy, relapse in acute myeloid leukemia treatment affects 40-50% of young people and a vast majority of old ones. Therefore, it is essential to develop effective and safe therapeutic approaches so as to deal with relapses of AML (8-10).

Indole compounds have continuously attracted the attention of researchers and emerged as a valuable research topic due to their exceptional therapeutic properties. These compounds are known as "premium scaffolds" owing to their ability to bind to multiple receptors with high affinity, which can potentially result in the creation of new bioactive drugs. Indole compounds have a wide range of biological activities that are relevant to a variety of human diseases. They are commonly used in the development of drugs and synthetic drug prototypes, including antitumor, antiviral, antimicrobial, and anti-inflammatory agents. Indole derivatives have also shown promising results for their cytotoxic effects on various types of cancer. In particular, recent studies have highlighted the potential of these derivatives to treat breast cancer, vulvar intraepithelial neoplasia, and human papillomavirus-induced cervical cancer (11-14).

The inhibitory effects of indole derivatives are regulated by various molecular pathways. Understanding the molecular mechanisms that contribute to the formation of leukemia and how they work together can potentially lead to the development of novel therapeutic approaches aimed at increasing the

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survival rate of patients (15). Apoptosis is the main form of cell death in normal and cancerous cells. This is a programmed process highly regulated by the BCL-2 family of proteins which include antiapoptotic proteins (BCL-2, BCL-XL, MCL-1, BCLW, and BFL-1) and proapoptotic proteins (BAX, BAK, and BOK). Evasion from apoptosis is a crucial step in tumor development, and cancer cells develop various mechanisms to avoid this process. The evidence suggests that apoptosis resistance occurs in almost all malignancies, but the underlying mechanisms vary depending on the type of tumor (16). In this regard, the overexpression of anti-apoptotic BCL-2 family members is relatively common in newly diagnosed cancer as well as in cases that has grown resistant to treatment (17). On the other hand, numerous tumor types have been shown to lose their pro-apoptotic BCL-2 members or keep them downregulated (18). The apoptotic effects of indole-3-carbinol (I3C) and 3,3′-diindolylmethane (DIM) were exhibited in prostate cancer cells (19) breast cancer (20) human cervical cancer (21) and hepatocellular carcinoma (22).

Certain effective drugs used in the treatment of cancer cannot be administered due to their inability to penetrate the cell membrane and precipitation in an aqueous medium. In such cases, the use of nano-carriers is a viable solution that eliminates this limitation. Several studies have been conducted to assess the impact of encapsulating various compounds, including indoles, on cancer cell lines and other diseases (23). The present study examined the effects of an indole derivative (2-AITFEI-3-C) in both free and liposomal forms on the expression of Bax and BCL-2 genes in the KG-1 cell line. Previous studies have investigated the effect of the free form of this indole compound (24-26), and the results of using free indole in this study are in line with them. This study has assessed the efficiency of an indole compound through loading it in liposome and compared it with the free form. The findings suggest that both free and liposomal indole may be effective in modulating the expression levels of BAX and BCL-2 genes in an AML cell line.

As the MTT assay results indicated, both free and liposomal types of indole were effective in inducing cytotoxic effects in the AML cell line over 24- and 48-hour periods. The lethal effective dose of the liposomal indole system was significantly lower than that of free indole. This suggests that low quantities of liposomal drugs can improve the anti-cancer efficacy while reducing drug toxicity and mitigating negative effects on healthy cells.

Specifically, treatment with both free and liposomal indoles led to a significant increase in the expression level of the BAX gene after 24 and 48 hours. This increase was more pronounced following the treatment with the liposomal indole system, which may suggest that the liposomal system is a more effective delivery vehicle for indole. Conversely, the treatment with free and liposomal indoles lowered the expression level of the BCL-2 gene after 24 and 48 hours, but a significant reduction occurred only after 48 hours of treatment. It is concluded that liposomal indole may better reduce BCL-2 expression compared to free indole. These findings combined with the observed changes in BAX and BCL-2 expression levels suggest that the liposomal indole system is a potential treatment option for AML.

The same studies were done to investigate the effect of this indole derivative in cancer treatment. In one study, the expression of self-renewal regulatory factors in a leukemia NB4 cell line was evaluated after treatment with this indole compound. A real-time PCR analysis

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indicated that the expression of NANOG/OCT4 was downregulated. In addition, a western blot analysis showed a significant decrease in the OCT4 expression (27). Some studies have looked into the effect of indole derivatives on cell proliferation. For example, Heydari et al. (2) and Karimabad et al. (24) examined how indole compounds impact the expression of apoptotic mediators at both the gene and protein levels. Based on their findings, it appears that incubation with such compounds leads to the downregulation of Bcl-2 and the upregulation of Bax. Other characteristics of such compounds have been reported in some studies. One study showed that the expression of SALL4 gene significantly decreased after treatment with an indole compound (28).

Generally, additional research is required to assess the safety and effectiveness of the liposomal indole system in both in vitro and in vivo settings. It is also necessary to determine an ideal dosage and administration schedule for the medication. This study offers valuable insight into the potential of indole as a treatment option for AML and emphasizes the importance of further exploration of its therapeutic possibilities.

Conclusion

Our findings suggest that free and liposomal indole compounds can induce the apoptosis of AML-KG1 cells. Moreover, liposomal indole has a greater inhibitory effect on the growth of KG1 cells than free indole. As the test results showed, the effect of liposomal indole after 48 hours of incubation was more significant than that in the initial 24 hours of incubation. Therefore, liposomal indole seems to be a promising effective anticancer medication, particularly for AML.

Ethics approval

The study was conducted in accordance with the ethics committee of Yazd Reproductive Sciences Institute, with the code of ethics "IR.RUMS.REC.1396.115".

Human and animal rights: No animals/humans were used in the experiments of this research.

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Footnotes

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None

Conflict of interests

There is no conflict of interests.

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