https://doi.org/10.31925/farmacia.2022.6.11 **ORIGINAL ARTICLE**

CYTOTOXICITY ASSESSMENT OF LIPOSOMES LOADED WITH BIOLOGICALLY ACTIVE SUBSTANCES ON ORAL TUMOUR CELLS

DENISA PREDUT [1#], ALEXANDRA DENISA SEMENESCU [3,4#], ANIKO MANEA [5]*, STEFANIA DINU [1], RAMONA POPOVICI [1], ANCA JIVANESCU [1,2]

[1]*Faculty of Dental Medicine, "Victor Babeş" University of Medicine and Pharmacy Timisoara, 2 Eftimie Murgu Square, 300041, Timişoara, Romania*
[2]*Research Centre of Digital and Advanced Technique for Endodontic, Restorative and Prosthetic Treatment (TADERP), 2 Eftimie Murgu Square, 300041, Timişoara, Romania*
[3]*Faculty of Pharmacy, "Victor Babeş" University of Medicine and Pharmacy Timişoara, 2 Eftimie Murgu Square, 300041, Timişoara, Romania*
[4]*Research Centre for Pharmaco-Toxicological Evaluations, Faculty of Pharmacy, "Victor Babeş" University of Medicine and Pharmacy Timişoara, 2 Eftimie Murgu Square, 300041, Timişoara, Romania*
[5]*Faculty of Medicine, "Victor Babeş" University of Medicine and Pharmacy Timişoara, 2 Eftimie Murgu Square, 300041, Timişoara, Romania*

**corresponding author: manea.aniko@umft.ro*
[#]*Authors with equal contribution.*

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Abstract

Lately, the scientific world has turned its attention to oral pathologies, observing the contribution of the health of the oral cavity to the general condition of the body. One of the most well-known diseases, oral carcinoma, is ranked worldwide as the sixth type of cancer in terms of frequency, and in Romania according to the 2020 statistics, it is on the 3rd place. The treatment generally consists in the administration of chemotherapy, hormonal therapy or surgical interventions, but these attract numerous adverse effects. Therefore, knowing the effectiveness of active principles extracted from plants in various pathologies, our research group wanted to incorporate betulin into liposomes to remove the inconveniences due to synthetic therapy. Likewise, encapsulation improves the solubility of the phytocompound. Morphological analysis revealed that the liposome-betulin system changes cell shape and confluence, the most visible effect being observed at 10 µM. MTT assay was performed to determine cell proliferation. Our results confirmed the activity of the liposome-betulin complex to inhibit cell viability from 75% at the lowest concentration (1 µM) to 29% at the highest concentration tested (10 µM). While betulin at 10 µM recorded a viability value of approximately 65%. In addition, the Hoechst assay established a dose-dependent tendency of betulin, and especially betulin incorporated into liposomes, to change shape and fragment cell nuclei.

Rezumat

În ultimul timp, lumea științifică și-a îndreptat atenția spre patologiile de la nivel oral, observându-se contribuția sănătății cavității bucale la starea generală a organismului. Una dintre cele mai cunoscute afecțiuni, carcinomul oral, este clasat la nivel mondial ca fiind al șaselea tip de cancer ca și frecvență, iar în Romania conform statisticilor din 2020 se află pe locul 3. Tratamentul constă în general în administrarea de chimioterapice, terapie hormonală sau intervenții chirurgicale, însă aceastea atrag după sine numeroase efecte adverse. Prin urmare cunoscându-se eficacitatea principiilor active extrase din plante în diverse patologii, grupul nostru de cercetare a dorit încorporarea betulinei în lipozomi pentru a înlătura dezavantajele datorate terapiei de sinteză. În plus, încapsularea îmbunătățește solubilitatea fitocompusului. Analiza morfologică a relevat faptul că sistemul lipozom-betulină modifică forma și confluența celulară, cel mai vizibil efect fiind observat la 10 µM. Analiza MTT a fost realizată pentru a determina proliferarea celulară. Rezultatele noastre au confirmat activitatea complexului lipozom-betulină de a inhiba viabilitatea celulară de la 75% la concentrația cea mai scăzută (1 µM) la 29% la cea mai mare concentrație testată (10 µM). Pe când betulina la 10 µM a înregistrat o valoare a viabilității de aproximativ 65%. În plus, testul Hoechst a stabilit tendința betulinei și mai ales a betulinei încorporate în lipozomi de a modifica forma și de a fragmenta nucleii celulari, dependent de doză.

Keywords: oral cancer, cytotoxicity, betulin, betulin-loaded liposomes

Introduction

Pathologies among the population are increasingly widespread, involving all age categories from new-borns to the elderly and affecting all systems and organs [1-4]. Over the past few years, medical research has placed greater emphasis on the branch of dentistry, which had been underestimated for many years. These studies have highlighted the critical role the oral cavity plays in the propagation of systemic diseases [5]. Oral health contributes to the general condition of the body and has a strong impact on the quality of life. Dental disorders, furthermore, to

causing pain, affect normal physical functions such as speaking and eating, and affect social life [6]. In general, oral diseases can be prevented, but they are very widespread throughout life and have significant adverse reactions on society. Dental conditions are chronic and progressive ailments. The most current pathology, tooth decay affects all categories of people from children to adults, being a lifelong condition. A wide variety of illness affect the soft and hard tissues of the oral cavity, including congenital anomalies, craniofacial disorders, wounds, and infections. General health is affected most often by dental caries, periodontal disease, and oral cancer [7]. Dental caries represents the destruction of enamel and dentin by the acid by-products resulting from the bacterial fermentation of sugars. Caries formation is a dynamic process, in which the stages of demineralization of the dental structure alternate with those of mineralization depending on the pH of the plaque biofilm [8]. Periodontal conditions are represented by chronic inflammation of the tissues that support the teeth. Initially, the disease can be manifested by gingivitis, inflammation of the soft tissues with bleeding and can evolve, in people with low immunity, to periodontitis, which over time destroys the support of the periodontal tissue and even the bone that surrounds the teeth [9].

Oral cavity carcinomas involve a wide category of neoplasms. Oral cancer is considered the sixth most common cancer worldwide [10]. Head and neck squamous cell carcinoma (SCC) comprise a whole set of epithelial cancers, such as pharynx, larynx, lips and salivary gland cancers [11]. According to the statistics from 2020, Romania is in 3rd place regarding the general rate of mouth and oral cancer [12]. Treatment generally consists of a combination of chemotherapy, immunotherapy, hormonal therapy and surgery, but these treatments produce many severe side reactions. Therefore, alternative methods are needed that have minimal adverse effects and limit the spread of cancer. Over time, plants and natural compounds have demonstrated their benefit in various acute and chronic pathologies, as well as in the treatment of oral cancer [13].

Pentacyclic triterpenes are a class of active principles intensively studied for their anticancer effect [14, 15], among the best known are betulin and betulinic acid found in the bark of *Betula* sp. [16]. However, the effectiveness of these phytocompounds is influenced by maintaining stability and bioavailability. Unfortunately, the bioavailability of betulin and betulinic acid is very low due to poor solubility in water. Thus, this inconvenience was removed either by obtaining complexes with hydrophilic substances, or by derivatization to more soluble molecules [17]. The incorporation of plant extracts and phytocompounds into various delivery systems has become a common practice in the medical industry, necessary to improve the solubility, stability and bioavailability of active compounds [18]. Furthermore, there are studies that prove that targeted delivery systems are promising in the treatment of various types of cancer [19, 20].

Lipid-based systems are rated as effective carrier systems in delivering natural compounds, due to the fact that lipids are biodegradable and can improve transcellular transport by disturbance lipid bilayers. The liposome is a colloidal, lipidic, delivery system that can encapsulate both hydrophilic and lipophilic compounds. Liposomal systems can help improve the solubility of active principles, reduce toxicity and targeted drug delivery [21]. In dentistry, liposomes have been used to prevent caries and gingivitis and in the treatment of oral injuries and periodontitis. Moreover, the potential of liposomal systems as drug carriers in the treatment of ulcerated oral mucosa was investigated *in vivo*, noting that liposomes decrease systemic drug concentration and increase local concentration [22].

The therapeutic effect of betulin and its derivatives has been tested *in vitro* on a large number of cell lines [23] and also, *in vivo*, on animal models [24, 25]. The concept of using nanoparticles to deliver the active substance to the diseased site has attracted much attention. Furthermore, it was discovered that the liposomal formulation of betulinic acid leads to a stronger antitumour effect, supported by reduced systemic toxicity, on lung and colon cancer [26]. However, the encapsulation of natural compounds in carrier systems remains little explored for the treatment of oral cancer.

The purpose of this study is to determine the efficiency of betulin incorporation in liposomes and to evaluate their cytotoxicity on the Detroit-562 pharyngeal cancer line. More precisely, it was desired to obtain a betulin release system at the level of oral cancer that would present a strong antitumour effect.

Materials and Methods

Reagents

Betulin (B9757 - 5G), cholesterol, lecithin, methanol, chloroform were purchased from Sigma-Aldrich Chemie GmbH (Darmstadt, Germany). For cell cultures dimethyl sulfoxide (DMSO - solvent), cell culture supplements – foetal bovine serum (FBS), penicillin, streptomycin; phosphate saline buffer (PBS), trypsin-EDTA solution and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability kit were purchased from Sigma-Aldrich, Merck KgaA (Darmstadt, Germany). Specific culture medium - Eagle's Minimum Essential Medium (EMEM-ATCC 30-2003™) was acquired from ATCC (American Type Cell Collection, Lomianki, Poland). All reagents were of analytical purity and for cell culture use.

Preparation and characterization of betulin liposomes

The thin-film dispersion method was used to obtain liposomes loaded with betulin. In short, the preparation

involved: betulin, phosphatidylcholine and cholesterol (ratio 0.3:5:1) were stirred in solvent media (methanol-chloroform, 1:1 v/v) for 60 min at 42°C; solvent mixture was removed with a rotary evaporator; the resulting lipid film was hydrated with phosphate buffer for 30 min at 42°C; reaction mixture was sonicated for 15 min and finally was filtered and maturated overnight. The control samples (without betulin) were similarly prepared.

The particle size and zeta-potential/stability of the samples were evaluated by DLS (Dynamic light scattering) and on Zetasizer (Nano ZS system, Malvern Instruments, Malvern, UK) following the methods described before [27].

For the encapsulation efficiency, the liposomes were spectrophotometrically analysed, the measurements were conducted after realization of the calibration curve, and the absorbance registered at room temperature on a T70 UV-Vis spectrophotometer (PG Instruments Ltd., Lutterworth, UK).

Cell lines

In vitro experiments were conducted on Detroit-562 (ATCC® CCL-138™) - pharyngeal carcinoma cells acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured and grown in specific culture medium – Eagle's Minimum Essential Medium supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin mixture (Pen - 100 U/mL/Strep - 100 g/mL). The experiments were carried out under standard condition: temperature 37°C and 5% CO_2.

Cell viability

Cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, Detroit-562 cells were seeded in 96-well plates (1×10^4 cells/well) and stimulated with three concentrations of Betulin, Liposome-Betulin and Liposome Blank (1, 5 and 10 µM) solubilized in DMSO for 24 h. A volume of 10 µL/well of MTT solution (5 mg/mL) was added in each well after 24 h. The cells were subsequently incubated for 3 h at 37°C, followed by addition of solubilization solution (100 µL/well) and kept at room temperature for 30 minutes in the dark. Finally, the absorbance measurement of the reduced MTT was performed at 570 nM and 630 nM using Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA). The experiments were carried out in triplicate and the results were indicated as cell viability percentage (%).

Cell morphology

To comprehend the cytotoxic effect of the tested compounds on Detroit-562 cells, microscopic evaluation of cells morphology and confluence was conducted after 24 h of stimulation. The impact of Betulin, Liposome-Betulin and Liposome Blank on cells was monitored using an Olympus IX73 inverted microscope (Olympus, Tokyo, Japan) and images were analysed using cellSens Dimensions v.1.8. Software (Olympus, Tokyo, Japan).

Nuclear staining

The Hoechst 33342 staining assay was conducted to highlight the cytotoxic effects of Betulin, Liposome-Betulin and Liposome Blank at the level of Detroit cell nuclei. The protocol was performed according to the manufacturer's instructions. The cells were cultured at 1×10^5 cells/well in 12-well plates and stimulated with increasing concentrations of test compounds (1, 5 and 10 µM). After 24 h, the cell medium was removed and 500 µL/well of 1:2000 staining solution diluted in PBS was added. After incubation at room temperature in the dark for 10 minutes, the staining solution was removed and washed three times with PBS. Staurosporine solution (5 µM) was used as positive control for apoptosis (incubation for 3 h at 37°C).

Data analysis

Results are expressed as ± standard deviation (SD). GraphPad Prism software version 9.3.1 for Windows (GraphPad Soft-ware, San Diego, CA, USA, www.graphpad.com) was utilized to analyse and present the statistical analysis data. One-way ANOVA test was realized to determine statistical differences between samples, followed by Dunnett's multiple post-test. Statistically significant differences between data were marked with: * $p < 0.1$, **** $p < 0.0001$.

Results and Discussion

It has been reported that betulin and its derivative, betulinic acid, have a wide range of anticancer effects. In particular, the antitumour activity against melanoma is known [28-31], but the latest data from the literature and the conclusions of our study support the fact that other types of cancer are also sensitive to betulin [32-34].

Due to the fact that betulin, betulinic acid and their derivatives have a low solubility in water, their administration *in vivo* is limited. To overcome this inconvenience, encapsulation in liposomes was used to improve its bioavailability and thus increase its therapeutic activity, a desired effect in our study as well [35].

Betulin-loaded liposomes presented a negative zeta potential, an average size and distribution of these types of nanoformulations, and a low polydispersity index (0.17 - control sample, 0.21 - loaded sample) homogeneous distribution and stability being confirmed. The encapsulation efficiency was greater than 45%. All these properties confirm the correct incorporation of the betulin into liposomes.

To examine the effects of the compounds on the level of pharyngeal carcinoma cells, MTT assay was used. In the case of the parent compound, Betulin, there is a decrease in cell viability depending on the concentration tested. Thus, at the concentration of 1 µM, the cell

viability was similar to that of the control cells, not stimulated. In contrast, at 5 μM and 10 μM concentrations, cell viability decreased to 73.5% and 65.3%, respectively. Regarding Liposomes Blank, a decreasing effect of cell viability can also be observed, but not stronger than in the case of Betulin and Betulin liposomes. Consequently, a concentration of 10 μM resulted in a 73% decrease in viability (Figure 1). Depending on the concentration tested, Liposome-Betulin exhibited a pronounced cytotoxic effect. In the case of the concentration of 1 μM, the effect of decreasing the cellular viability was not a major one, the viability having the value of approximately 75%. However, higher concentrations resulted in a marked decrease in the number of viable cells, to a cell viability value of about 29% at a concentration of 10 μM (Figure 1).

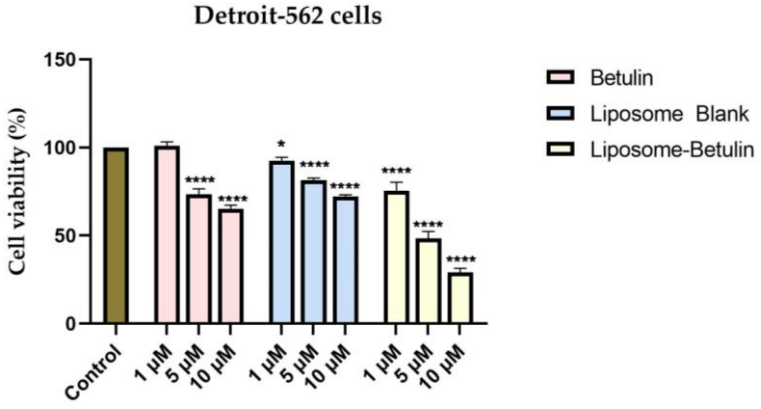

Figure 1.

In vitro viability evaluation of Betulin, Liposome Blank and Liposome-Betulin (1, 5 and 10 μM) in Detroit-562 cells at 24 h post-stimulation by MTT assay

The results are expressed as cell viability percentage normalized to control cells (unstimulated). The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was applied to determine the statistical differences in rapport with control cells followed by Dunnett's multiple comparisons post-test (* $p < 0.1$, **** $p < 0.0001$).

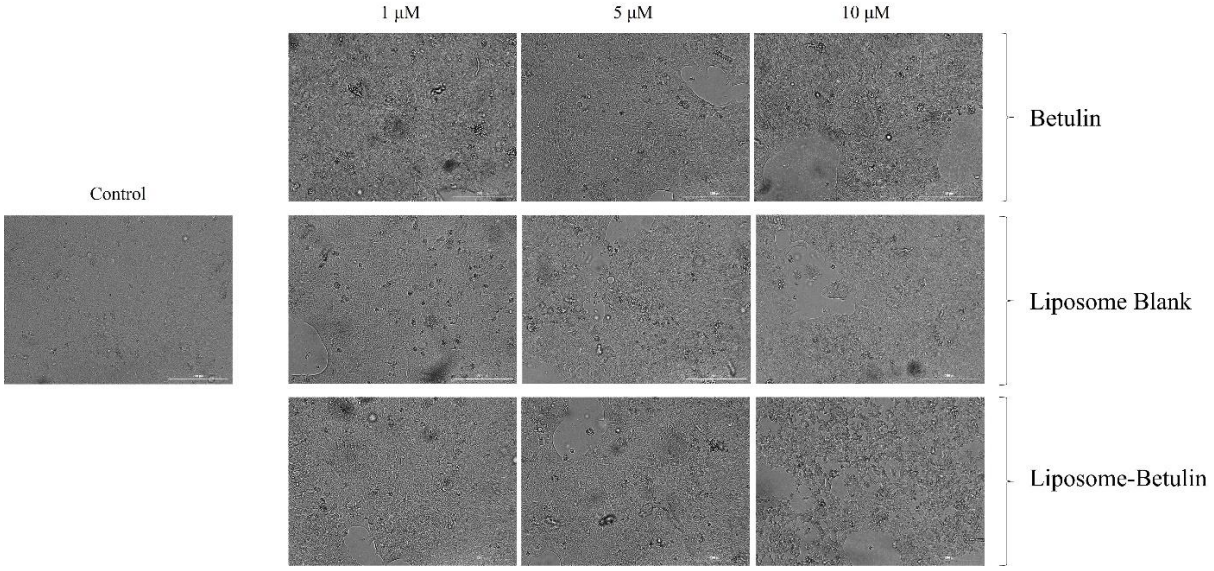

Figure 2.

Morphology and confluence of Detroit-562 cells following the 24 h treatment with betulin, liposome-betulin and liposome blank (1, 5 and 10 μM). The scale bars indicate 200 μm

An analysis of the cell morphology was performed after 24 hours of stimulation to provide an overview of the effects of the three samples on pharyngeal carcinoma cells. Betulin, at a concentration of 1 μM, did not produce significant changes in cell morphology, the cells remaining similar to control cells, unstimulated. In high concentrations, a decrease of cell confluence was observed, depending upon the concentration tested. Consequently, the most intense morphological changes were observed at the highest concentration (10 μM) – rounding of cells, separation of cells from the plaque, and a decrease in cell confluency.

Liposome-Betulin, when tested at a concentration of 1 μM, did not cause major changes in cell morphology. In contrast, higher concentrations resulted in a significant decrease in confluence and number of cells. Furthermore, the structure of the cells was evidently altered, particularly at the concentration of 10 μM, when signs of cell death were noted, with the cells becoming round and detached from the plaque. In terms of Liposome Blank's impact on morphology, a slight decrease in cell confluency and an alteration of cell shape were mainly associated with the 10 μM concentration. Therefore, the lowest concentration (1 μM) did not have a major impact on the number of cells or the morphology of cells (Figure 2).

In light of the results obtained previously, namely that the tested compounds had a cytotoxic effect on Detroit-562 cells, the next step in the study was to evaluate the impact on the nuclei of the cells.

In the case of betulin, there was a decrease in the number of nuclei with increasing concentration. Furthermore, it was observed that at a concentration of 5 μM, signs of cellular apoptosis were present, such as nucleus condensation and the appearance of apoptotic bodies. The strongest signs of apoptosis were observed at a concentration of 10 μM.

Upon application of liposomes-betulin, the number of nuclei significantly decreased, resulting in visible alterations. Consequently, all concentrations tested caused a strong condensation of chromatin, nuclear fragmentation, as well as the formation of apoptotic bodies. A concentration of 10 μM led to a massive reduction in the number of nuclei, these becoming very condensed, a characteristic sign of apoptosis.

By contrast, liposome blank caused no significant changes at the level of the nuclei, with only a slight condensation of chromatin being recorded at the 10 μM concentration (Figure 3).

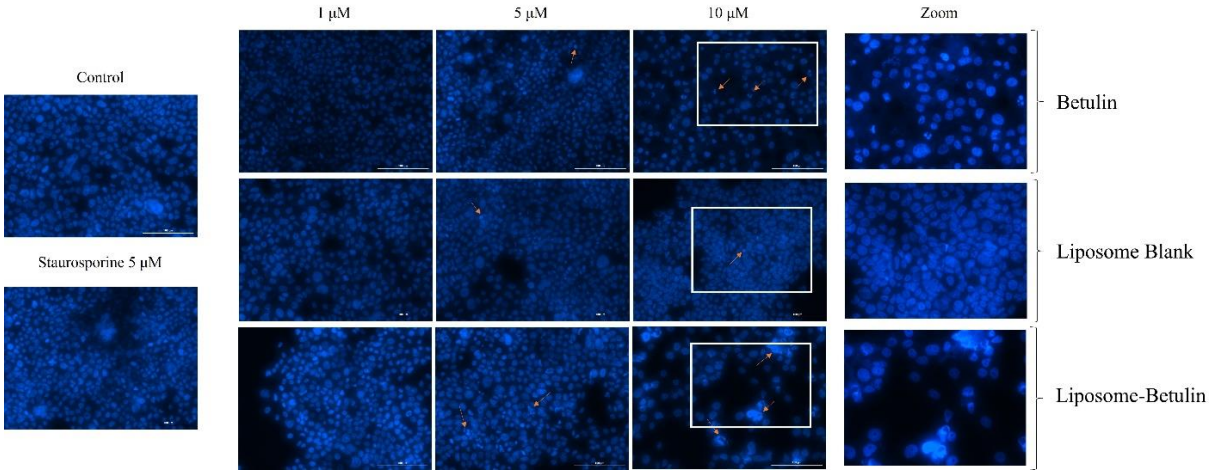

Figure 3.

Detroit-562 nuclei stained with Hoechst 33342 dye after a 24 h treatment with betulin, liposome-betulin and liposome blank (1, 5 and 10 μM). Staurosporine (5 μM) was used as the positive control for apoptotic changes at nuclear level. The orange arrows indicate signs of apoptosis. The scale bars represent 100 μm

We can observe in the literature the fact that betulin and its derivatives have been studied regarding the antitumour effect on a variety of cancer cell lines, not having completely touched the subject regarding its effectiveness in oral cancer. Our results are in line with the published data regarding cytotoxicity analyses: viability, morphology and apoptosis.

The study led by Csuk evaluated the antitumour effect of several betulin derivatives on 15 human cancer lines. Moreover, the anticancer effect of the first synthesized compound encapsulated in liposomes was investigated, using the sulforhodamine B (SRB) assay. Betulin derivatives showed a remarkable cancer activity on several cell lines such as HCT-116, MCF-7, SW-1736, A549, A2780 but also on head and neck tumour lines (FADU and A253). Following the data obtained, it was evident that in most cases the betulin derivative incorporated in the liposomal delivery systems has a stronger cytotoxic effect than the basic compound, this being a first step towards the incorporation of more triterpenic compounds and the evaluation of the antitumour effect. Treatment of submaxillary salivary gland cancer cells (A253) with up to a maximum concentration of 30 μM of the tested compounds resulted in IC50 values of 11.1 μM for betulinic acid, 2.7 μM for compound 1 with an acetylenic chain in the C-28 position and respectively 3.6 μM for the derivative incorporated into liposomes [30]. In the case of the FADU cell line, of pharyngeal carcinoma, a stronger cytotoxicity was observed with an IC50 value of 10.4 μM for betulinic acid, 5 μM for the derivative and respectively 4 μM for the derivative incorporated in the liposomal system [36].

The research group conducted by Lv reported the effect due to the liposomal incorporation of paclitaxel and ursolic acid on HSC-3 human head and neck

cancer cell lines. The combination of the natural triterpene compound and the anticancer drug was pursued to reduce the toxicity of paclitaxel and to increase its anticancer action. The results suggest that the liposomal system with the two incorporated substances (UA-PTX-LiP) has a greater cytotoxic effect than pure or liposome-incorporated paclitaxel in a dose-dependent manner (0.001, 0.01, 0.1, 1 and 10 μg/mL). Likewise, it was observed that UA-PTX-LiP inhibited the growth of HSC-3 cancer cell line and increased cellular uptake that caused cell apoptosis more strongly than the chemotherapic administered alone or encapsulated. The more pronounced effect of the complex is believed to be due to the triterpene, ursolic acid, which is known to cause cancer cell apoptosis by inhibiting cyclo-oxygenase 2 [37].

Another study, which evaluated the antitumour activity of natural compounds encapsulated in liposomes on head and neck cancer cell lines, was carried out by Zheng *et al*. In this research, it was determined that the encapsulation of resveratrol in liposomes enhances the cytotoxic effect of the flavonoid on the SCC-VII cell line derived from murine squamous cell oral cancer. Moreover, the conjugation of nanoparticles with a dodecapeptide (GE11) and the incorporation of resveratrol (RSV-GL) specifically increased the antitumour effect, which was attributed to the active internalization mediated by specific receptors of the complex, which triggered the binding of GE11 peptides to cancer cells overexpressing the receptor epidermal growth factor. In addition, the flavonoid encapsulated in liposomes induced an increased apoptotic effect of ~ 17.5% in early apoptosis and ~ 4% in late apoptosis, while RSV-GL determined a stronger apoptotic effect of ~ 60% and ~ 5% respectively. RSV-GL also demonstrated the highest antitumour efficacy *in vivo*, with a 2-fold decrease in tumour volume in female BALB/c nude mice. We can state that the development of the system of liposomes conjugated with EGFR targeted peptides for the delivery of natural compounds can be an important step in reducing the development of oral carcinomas [38].

Other group of researchers showed the efficacy of encapsulated natural compound therapy, more exactly of liposomal curcumin in suppressing the spread of head and neck cancer, on CAL27 and UM-SCC1 cancer cell lines in a dose-dependent manner (25 - 400 μmol/L). Treatment with the liposomal complex repressed nuclear factor κB activation without affecting phospho-AKT expression. *In vivo*, tumour xenografts from nude mice were stifled after 5 weeks of intra-venous administration of liposomal curcumin, without any evidence of toxicity following autopsy [39].

The obtained results are important evidence, which are close to achieving the goal of long-term oral cancer treatment, the use of an alternative treatment modality with minimal adverse side effects.

Conclusions

The therapeutic actions of phytocompounds are well known in various pathologies, but most of the time natural compounds cannot be administered due to their low solubility, and it is necessary to find appropriate formulations to increase their biomedical properties. Our study demonstrated the efficiency of incorporating betulin into liposomes and highlighted the strong cytotoxic effect of the liposomal complex on Detroit-562 pharyngeal cancer cells through the analyses of morphology, viability and nuclear staining. Depending on the concentrations tested, liposome-betulin showed a pronounced cytotoxic effect, with a decrease in cell viability of 75% at the concentration of 1 μM and of 29% at the highest concentration, 10 μM. Besides, at 10 μM, changes in cell morphology were observed with signs of cell death, the cells becoming round and then detached from the plate. Also, the liposomal system significantly decreased the number of nuclei, causing nuclear fragmentation and the formation of apoptotic bodies.

Conflict of interest

The authors declare no conflict of interest.

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