CHAPTER 8

UPTAKE STUDIES OF FREE AND LIPOSOMAL SCLAREOL BY MCF-7 AND H-460 HUMAN CANCER CELL LINES

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Abstract: The aim of this study was to investigate the uptake of free and liposomal sclareol and its effect on the growth inhibiting activity against MCF-7 and H-460 human cancer cell lines in vitro. Liposomes composed of EPC/DPPG at molar ratio 9:0.1, used to incorporate sclareol, were prepared by the thin-film hydration method followed by sonication. The final liposomal preparation (EPC/DPPG/Sclareol 9:0.1:5 molar ratio) as well as free sclareol (100μM) were incubated up to 96 hours with both cell lines. Sclareol was extracted from cells using the Bligh-Dyer method and was measured by HPTLC/FID. The results showed that the uptake of free sclareol by both cell lines was faster and higher compared to that of its liposomal form. In both cell lines, free sclareol showed high cytotoxicity, while the liposomal sclareol showed reduced cytotoxicity without affecting its ability to reduce the cell growth rate. These findings suggest that liposomal sclareol may possess chemotherapeutic advantages over its free form and can be used for future in vivo experiments for the treatment of these two types of human cancer

Keywords: Sclareol, liposomes, cytotoxicity, uptake, breast cancer, lung cancer

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Abbreviations: EPC: egg- phosphatidyl choline; DMSO: dimethyl sulfoxide; DPPG: dipalmitoyl phosphatidylglycerol; HPTLC/FID: High Performance Thin Layer Chromatography/Flame Ionization Detector; NCI: National Cancer Institute; NIH: National Institutes of Health, RPMI: Roswell Park Memorial Institute

1. INTRODUCTION

The most common types of cancers in adults are: breast, lung, colon and prostate cancer. Early diagnosis and prompt treatment including chemotherapy still hold out the hope of long-term survival. Breast cancer in women is the leading cause of death in women aged 35–54. Metastases to lung, liver, bone marrow, brain and other sites is the reason of death. Drug therapy for breast cancer includes cytotoxic agents among others like hormonal agents (Pratt *et al*. 1994). Lung cancer is divided into two major types; non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). SCLC differs from NSCLC in that it grows rapidly and responds better to chemotherapeutic agents. NSCLC is heterogeneous aggregate of at least three distinct histologies of lung cancer including epidermoid or squamous carcinoma, adenocarcinoma and large-cell carinoma (Pakunlu *et al*. 2004). It grows slowly and does not respond well to chemotherapy. Treatment depends on a number of factors, including the type of lung cancer (non-small or small cell lung cancer), the size, location, and extent of the tumor, and the general health of the patient. Many different treatments such as surgery, chemotherapy, radiation therapy, photodynamic therapy, and combinations of them may be used to control lung cancer, and to improve quality of life by reducing symptoms (http://www.cancer.gov).

In anticancer therapy and particularly in chemotherapy, side effects depend mainly on the specificity and the dose of the drug used. The anticancer molecules used, due to their cytotoxicity, affect cancer cells and at the same time other cells that divide rapidly (http://www.cancer.gov). Nanotechnology can provide benefits in anticancer chemotherapy by increasing the specificity of drugs and delivering the bioactive molecules to the target site, hence reducing their toxic side effects. The delivery of cytotoxic molecules to tumor cells is an important aspect in the area of anticancer therapy and several delivery systems have been used as adequate for improving the delivery of biologically active molecules to target cells (Books *et al*. 2005; Gupta *et al*. 2005).

In the literature there have been many reports on the use of phospholipid vesicles as carriers for introducing biologically active substances into cells *in vitro* and *in vivo* (Allen *et al*. 1981). Liposomes are nowadays considered as non-toxic lipidic drug carriers and have been proven to be an adequate drug delivery system for lipophilic compounds since they can modulate the pharmacokinetic properties of the encapsulated drugs towards a more beneficial and safer use (Allen *et al*. 1999; Drummond *et al*. 1999). Liposomes or lipid vesicles are spherical self-closed

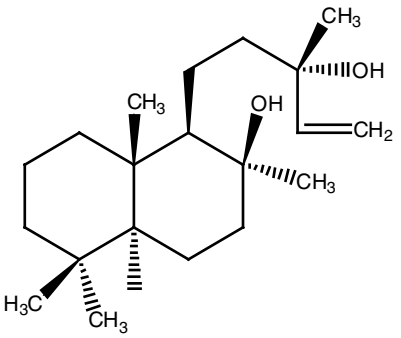


Figure 1. Chemical structure of sclareol

structures composed of curved lipid bilayers, which entrap part of the aqueous medium in which they freely float into their interior. The accumulating evidence from the studies of liposome-cell interactions indicates that liposomes are capable of interacting with cells via several mechanisms occurring simultaneously (Allen *et al*. 1981).

Sclareol (Figure 1) is a labdane diterpene with a structure of a ditertiary alcohol and is found in several plant species (Demetzos *et al*. 2001, 1999, 1990). In previous studies, sclareol exhibited significant cytostatic and cytotoxic effects, mainly in vitro, against several cancer cell lines derived either from leukemia or from solid tumors. It was furthermore found that the compound induced cell cycle arrest and apoptosis, while down regulating the expression of the protooncogene *c-myc*, (Dimas *et al*. 2001, 1999, 1998). Despite its interesting pharmacological actions, sclareol presents high lipophilicity. Additionally, in an attempt to evaluate the anticancer efficacy in vivo, free sclareol found to exhibit significant toxicity when administered intraperitoneally in immunodeficient mice. On the contrary using liposomes we were able to administer in a single cycle a total dose of 1100mg/kg in HCT116 xenografted NOD/SCID mice, which resulted in a significant regression of the tumors (Hatziantoniou *et al*. 2006).

The present study investigates the *in vitro* cytotoxicity of free and liposomal sclareol and the effect on growth rate, based on its uptake by two types of human cancer cells (*i.e*. MCF-7 and H-460).

2. MATERIALS AND METHODS

2.1. Materials

Both cell types (MCF-7 and H-460 cell lines) derived from human tumours, obtained from the NCI (NIH, USA). RPMI 1640, trypsin, L-glutamine, antibiotics, phosphate buffered saline (PBS) and foetal calf serum (FCS) were purchased from Euroclone, U.K. Dyes, salts and buffers as well as sclareol were purchased from Sigma

(Sigma Hellas, Athens). Egg-PC was purchased from Lipoid (Ludwigshafen, Germany), DPPG from Avanti Polar Lipids, Inc. (Alabastar, Alabama, USA) and sucrose from Sigma (St. Louis, MO, USA). All solvents (methanol, ethanol, acetic acid, DMSO) were of analytical grade and purchased from Labscan Ltd. Ireland.

2.2. Methods

Cell culture: Monolayer cultures of MCF-7 and H-460 were adapted to grow in RPMI 1640 medium, supplemented with 5% heat-inactivated FCS, (Euroclone, U.K.), 2 mM L-glutamine and antibiotics (100IU/mL penicillin and 100μg/mL streptomycin). Cells were incubated at 37°C, in a humidified atmosphere with 5% CO_2 (Celis 1994).

Determination of MCF-7 and H-460 cell growth rate: Prior to the application, the lyophilised liposomes were resuspended in deionised water. Free sclareol was diluted in DMSO at a stock of 20mM and kept at 4°C under lightproof conditions. Both were further diluted in supplemented RPMI at a final concentration of 100μM sclareol. Control cultures, in the presence of either DMSO or lipids were added in medium and were run in parallel. No differences in the growth of cells compared to untreated cells were observed in both cases (results not shown). Cells were cultured at plating densities of $3.7*10^6$ and $5*10^6$ cells/dish for H460 and MCF7 respectively, according to their doubling time, for 24h (adaptation time) prior to addition of the drug. After drug addition, the dishes were incubated up to 96h at predetermined time intervals (2, 4, 8, 16, 24, 48, 72 and 96 h). Control cultures received no drug. Cells were then trypsinized and counted using the Trypan blue dye exclusion method (Green and Moehle 1999). The cell growth rate was calculated according to the equation: $(T–C_0/U–C_0)*100$ when $T \geq C_0$ or $(T–C_0/C_0)*100$ when $T < C_0$, where C_0 is the number of viable cells right before adding the drug, T is the number of viable cells treated with sclareol and U is the number of viable cells for the untreated cultures. In that way negative numbers denote cytotoxic activity (Hatziantoniou *et al*. 2006).

Liposome preparation: EPC/DPPG liposomes were prepared by the thin-film hydration method (Hatziantoniou *et al*. 2006). The lipid film was prepared by EPC:DPPG:Sclareol 9:0.1:5 molar ratio and dried under vacuum for 12 h. Multi-lamellar vesicles (MLVs) were prepared by hydrating the lipid film with 0.15 M sucrose (sucrose to lipid ratio 2.24 w/w), above the gel to crystalline phase transition of the lipid (41°C), and stirring for 1 h. The resultant liposomal suspension was subjected to sonication for two 5 min periods interrupted by a 5 min resting period, in an ice bath using a probe sonicator (amplitude 100, cycle 0.7 – UP 200S, dr. hielsher GmbH, Berlin, Germany). The liposomal suspension was allowed to anneal any structural defects for 30 min and was centrifuged in order to separate the Small Unilamellar Vesicles (SUVs) from MLVs and from the titanium particles contributed from the sonicator probe. Subsequently, the liposomal suspension was freeze-dried and stored at 4°C. Size and ζ-potential of liposomes are the parameters that indicate their physical stability. 100 μl of the liposome dispersion was diluted

10-folds in HPLC-grade water (pH 5.6–5.7) immediately after preparation and mean z-average and ζ-potential of the empty and loaded SUVs were measured in order to determine the effect of sclareol loading on liposomal size and ζ-potential. Samples were scattered (633 nm) at a 90° angle and measurements were made at 25°C in a photon correlation spectrometer (Zetasizer 3000HS, Malvern Instruments, Malvern, UK) and analysed by the CONTIN method (MALVERN software).

The amount of drug trapped in liposomes was evaluated by HPTLC/FID (latroscan MK-5 new, latron Lab. Inc., Tokyo, Japan) (Hatziantoniou and Demetzos 2006; Hatziantoniou *et al*. 2006). Freeze-dried liposomal preparations were reconstituted to half of the initial volume by adding HPLC-grade water, resulting in a sucrose concentration of 300mM. The size and the ζ-potential of reconstituted liposomes were measured as described above. Samples were allowed to anneal for a period of 30 min prior to preparation of the diluted samples in RPMI growth medium.

Sclareol uptake: After treatment of cells up to 96h with free and liposomal sclareol and determination of the cell's growth rate, as noted above, sclareol was extracted from cells by the Bligh-Dyer method (Bligh and Dyer 1959), using $CHCl_3/CH_3OH/H_2O$. The sclareol cellular concentration was determined using HPTLC/FID (Iatroscan MK5^{new}; Iatron Lab. Inc., Tokyo, Japan), according to a calibration curve that was previously set up. Hydrogen flow rate was 160mL/min, airflow rate was 1900mL/min, and the scan speed was 30s/scan. As stationary phase silica gel sintered on quartz rods (Chromatorods-SIII; Iatron Lab. Inc.) was used in sets of ten rods (Hatziantoniou and Demetzos 2006; Hatziantoniou *et al*. 2006; Paradissis *et al*. 2005). All results were from three independent experiments. Statistical analysis, for all cell experiments, was done using the Student's t-test. A difference was considered significant if $p<0.05$.

3. RESULTS

The effect of free and liposome-incorporated sclareol on the growth rate of MCF-7 and H-460 cell lines are presented in Figure 2. As it is depicted in Figure 8-2A, free sclareol found to be highly cytotoxic for both cell lines. The growth rate reduced as early as 8 hours upon addition of sclareol.

Liposomal sclareol was substantially less cytotoxic than free sclareol at the same final concentration (100μM), which showed cytotoxicity after 48 hours of continuous incubation of cells. However, as it is clearly represented in Figure 2B, liposomal sclareol significantly reduced the growth rate of cells 24 hours later up on drug's addition. Measurements of sclareol content taken up by both cell types revealed that in the case of free sclareol at the time point that the growth rate was highly reduced (8 hours upon addition of sclareol), cells have already taken up the maximum amount of the drug (Figure 3A). Uptake of free sclareol from cells declined from that time point on and 48 hours later was diminished. The incorporation of sclareol into liposomes resulted in a slower rate of uptake from both cell lines (Figure 3B). The peak of the liposomal sclareol uptake was at 48 hours of incubation for MCF-7 cell line and 72 hours of incubation for H-460 cell line. After that the uptake is declined in both cell lines (Figure 3B).

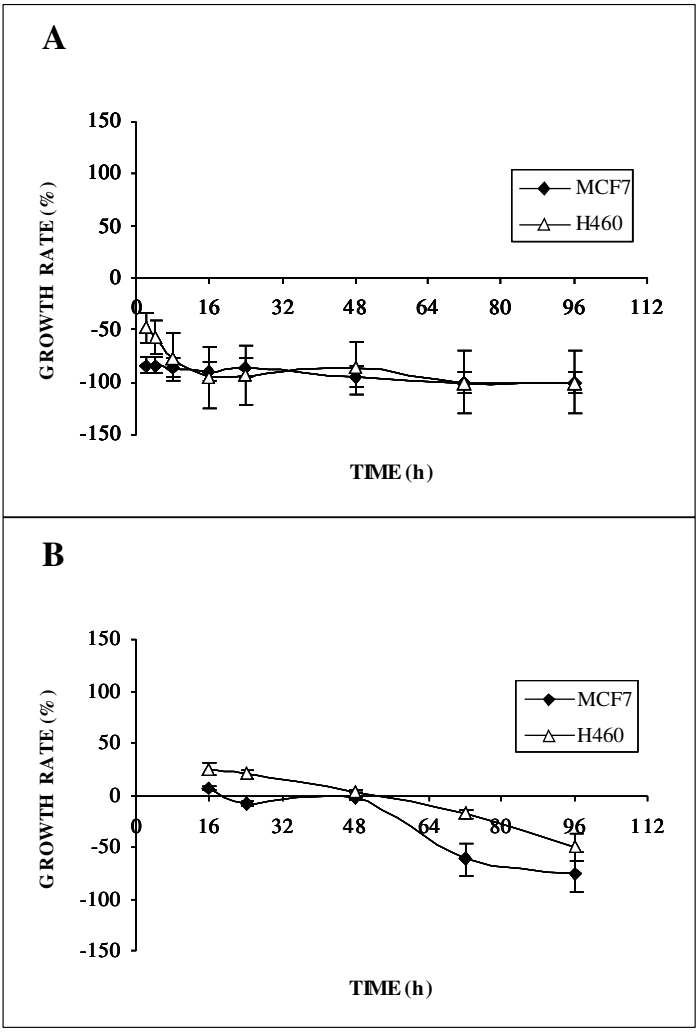
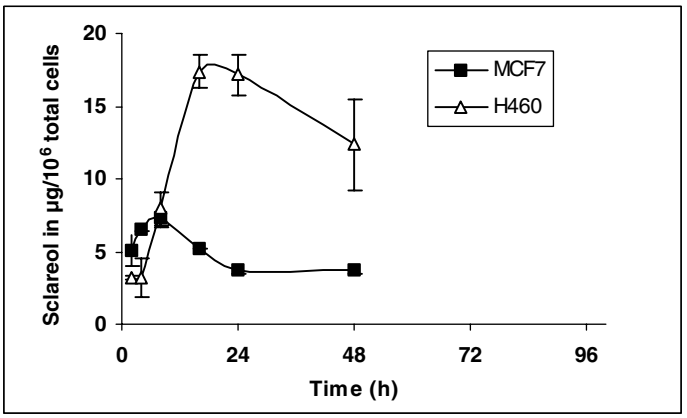


Figure 2. A: Effect of free sclareol on cell growth rate of MCF-7 (black diamonds) and H-460 (triangles) cell lines. Cells were incubated with 100μM of free sclareol. B: Effect of liposomal sclareol on cell growth rate of MCF-7 (black diamonds) and H-460 (triangles) cell lines. Cells were incubated with 100μM of liposomal sclareol

4. DISCUSSION

Extensive literature on the interactions of liposomes with cells has been accumulating over the past several years. However, due to the complex nature of liposome-cell interactions, interpretation of experimental results in terms of liposome-cell interactions has proven to be difficult. None of the mechanisms such as endocytosis,

A



B

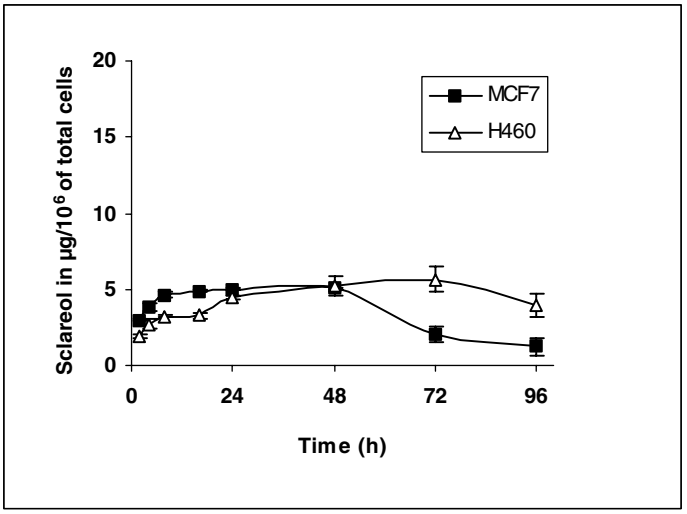


Figure 3. A: Uptake of free sclareol by MCF-7 (black cubes) and H-460 (triangles) cell lines. Cells were incubated with 100µM of free sclareol. B: Uptake of liposomal sclareol by MCF-7 (black cubes) and H-460 (triangles) cell lines. Cells were incubated with 100µM of liposomal sclareol

fusion or absorption of liposomes to cells, which are involved in liposome-cell interactions, are mutually exclusive (Allen *et al.* 1981).

Allen and co-workers (1981) have previously reported that liposome incorporated methotrexate, when tested in cell lines (EMT6 and S49), reduces and mediates the cytotoxicity of the free drug, via the uptake of free drug leaked from liposomes. In another study on the effect of liposomal daunorubicin against leukaemic cells, it has been reported that liposomal daunorubicin was devoid of acute effects such

as ROS production and ATP depletion that resulted in increased necrotic cell death (Liu *et al*. 2002). However, studies on the uptake of cytotoxic compounds by cells are of considerable importance.

Recently published results from our research group showed that sclareol might possess interesting pharmacological properties as it revealed significant cytostatic and cytotoxic effects against leukemic and solid tumor cell lines (Dimas *et al*. 2001, 1999; Hatziantoniou *et al*. 2006). It has been further demonstrated that sclareol induces cell cycle arrest at G0/1 phase of the cycle and kills cells via the mechanism of apoptosis (Dimas *et al*. 2001, 1999). When tested against colon cancer (HCT-116) xenografts developed in NOD/SCID mice, sclareol also exhibited a significant tumor regression in its liposomal form, while the free compound was highly toxic for animals, leading them to death (Hatziantoniou *et al*. 2006). In continuation of our research on sclareol, this work was focused on determining the effect of free sclareol on cell growth rate of human breast (MCF-7) and lung cancer (H-460) cell lines as well as the role of liposomes to alter the pharmakokinetic parameters of sclareol due to its different rate of uptake by cells. The results showed that liposomal sclareol was less cytotoxic at the concentration of 100μM than that of free sclareol at the same final concentration. At that concentration, free sclareol reduced the growth rate of cells while its incorporation into liposomes largely delayed the appearance of cytotoxic effects for both cell lines These experiments revealed that the reduced appearance of cytotoxicity of the liposomal sclareol could be well correlated with a lower accumulation rate of sclareol into cells (Figure 3B).

5. CONCLUSION

The present study was focused on the uptake of a bioactive compound namely sclareol by MCF-7 and H-460 human cancer cell lines. According to the findings, it has been shown that the liposomal sclareol retains significant growth inhibiting activity and alters the pharmacokinetic parameters. These results should be taken into account in feature *in vivo* studies.

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