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## Subcellular targeting for improved drug action : incorporation of sclareol into mitochondria targeted liposomes

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**SUB-CELLULAR TARGETING FOR IMPROVED DRUG  
ACTION: INCORPORATION OF SCLAREOL INTO  
MITOCHONDRIA TARGETED LIPOSOMES**

THESIS PRESENTED

BY

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## **Abstract**

The last century has witnessed dramatic advances in targeted delivery of therapeutic molecules. Current drug delivery approaches have successfully achieved tissue-specific and even cell-specific delivery of therapeutic molecules. However, in many cases such advances have resulted in less than the expected dramatic improvement in drug action. It is likely that in such cases, in spite of cell specific delivery, the drug molecules were not successfully delivered to their sub-cellular target inside the cell. It would appear reasonable that for the desired improvement in biological action of such a drug it is necessary to control the sub-cellular distribution of the drug molecule.

To achieve such sub-cellular targeting, we have been exploring the use of subcellularly targeted lipid-based nanocarriers. We have already shown that liposome's surface, modified with mitochondriotropic stearyl triphenylphosphonium (STPP) cations, target mitochondria [1]. We also demonstrated the potential of such a targeted delivery system in enhancing the therapeutic outcome of a drug molecule known to act on mitochondria when incorporated into mitochondria-targeted nanocarriers [2].

We studied the effect of sub-cellular targeting on the proapoptotic and cytotoxic action of sclareol. Sclareol (labd-14-ene-8, 13-diol) is a ditertiary alcohol, a member of the labdane type diterpenes with demonstrated antitumor activity by virtue of its proapoptotic action on tumor cell lines [3, 4]. The apoptotic action of sclareol is mediated in part by activation of the mitochondrial apoptosis pathway [4]. As sclareol acts on

mitochondria, we hypothesized that sclareol incorporated in mitochondria targeted liposomes will improve its proapoptotic activity and cytotoxic action on cancer cells.

We successfully incorporated sclareol into non-targeted liposomes and mitochondria targeted liposomes and assessed the *in vitro* cytotoxic activity and proapoptotic activity of sclareol incorporated in mitochondria-targeted liposomes compared to sclareol incorporated in non-targeted liposomes. Results from liposomal characterization showed preparations of stable liposomal formulations. A cell proliferation assay showed a significant increase in cytotoxicity of sclareol when incorporated into mitochondria-targeted liposomes compared to non-targeted liposomes. Using nuclei condensation by staining DNA of treated cells using Hoechst dye, we found a significant number of cells having apoptotic nuclei at 48 hrs when treated with sclareol incorporated into non-targeted liposomes. Nuclei condensation is a late apoptotic event which provided a time line to detect early apoptotic events such as caspase activation and detection of apoptotic cells by flow cytometry. As sclareol induces apoptosis partly via mitochondrial pathway, we measured the activation of mitochondrial pathway by measuring caspase-9 activation at 24 hrs. There was a significant increase in activation of caspase-9 in cells treated with sclareol incorporated into targeted liposomes compared to cells treated with sclareol incorporated into non-targeted liposomes. This increase in activation of caspase-9 reflected the molecular targeting of sclareol to its subcellular site of action mitochondria. Also, the cells undergoing apoptosis were counted by Vybrant® Apoptosis assay kit #3 from invitrogen using flow cytometry and showed an increased

number of cells undergoing apoptosis when treated with sclareol incorporated into targeted liposomes compared to sclareol incorporated into non-targeted liposomes.

In our earlier studies, we have already shown potential of mitochondria-targeted liposomes in delivering ceramide, drug known to act on mitochondria, to its subcellular site of action [2]. In the present study, we found that sclareol incorporated into mitochondria-targeted liposomes exhibits increased cytotoxic and proapoptotic activity compared to sclareol incorporated into non-targeted liposomes. By doing so, we demonstrated broad applicability of mitochondria targeted liposomes in delivering therapeutic molecules with diverse physicochemical properties to its subcellular target site of action.

**NORTHEASTERN UNIVERSITY**  
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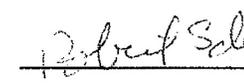
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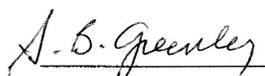
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## **OBJECTIVES AND SPECIFIC AIMS**

### **RATIONALE: -**

For any effective therapy, an adequate concentration of drug at the molecular target site needs to be achieved. Significant efforts have been made over the last three decades to develop drug carrier systems which are specific for certain tissues and cells. Even after achieving tissue accumulation and cell specific delivery, it failed in many cases to produce dramatic improvement in therapeutic action. It seems reasonable that it is also necessary to achieve the efficient delivery of a therapeutic molecule to its sub-cellular site of action for the desired improvement in its action. The development of drug delivery systems able to specifically reach sub-cellular sites has thus emerged as a new frontier in modern drug delivery approaches.

### **LONG TERM OBJECTIVES: -**

The long-term objective of this study was to develop subcellular targeted nanocarrier systems to specifically deliver drug molecules to their subcellular site of action and thereby improve therapeutic effect.

### **HYPOTHESIS: -**

Sclareol incorporated in mitochondria targeted liposomes will exhibit improved pro-apoptotic and cytotoxic action on cancer cells *in vitro*.

To test our hypothesis, the following specific aims were pursued.

### **SPECIFIC AIMS: -**

1. To measure and compare the cytotoxic activity using cytotoxicity assays in COLO205 cells when treated with sclareol incorporated in non-targeted liposomes and sclareol incorporated in targeted liposomes.

2. To perform Nuclei morphology assay in COLO205 when treated with sclareol incorporated in non-targeted liposomes.
3. To measure and compare the caspase activity of caspase-8 and caspase-9 at 24 hrs in COLO205 when treated with sclareol incorporated in non-targeted liposomes and sclareol incorporated in targeted liposomes.
4. To detect and compare percent cells undergoing apoptosis by flow cytometry in COLO205 when treated with sclareol incorporated in non-targeted liposomes and sclareol incorporated in targeted liposomes.

## **BACKGROUND AND SIGNIFICANCE**

Specific delivery of a therapeutic molecule to its sub-cellular site of action will dramatically improve its action [5]. Advances in targeted therapy have led to drug formulations with improved tissue-specific as well as cell-specific accumulation of therapeutic molecules in the hope of improved efficacy and reduced side effects [6]. Despite such efforts, tissue accumulation and cell-specific delivery resulted in less than expected dramatic improvement in drug action. A likely reason for such outcomes might lie in the fact that many drugs act on molecular targets associated with certain organelles inside mammalian cells [7]. So, despite successful cell-specific delivery or even cytosolic internalization, drug action may not improve if the drug molecule is unable to interact with its specific sub-cellular target site. It therefore becomes important to also address the sub-cellular targeting of a therapeutic molecule in any strategy designed to increase the therapeutic effect. Sub-cellular, i.e. organelle-specific drug delivery has thus emerged as the new frontier in modern drug delivery approaches [8].

Initial approaches to achieve sub-cellular targeting have involved chemical conjugation of targeting ligands to the therapeutic molecule. Peptide leader sequences, cell-penetrating peptides as well as organic molecules have been used to deliver a conjugated therapeutic molecules to sub-cellular structures like the nucleus and mitochondria [9-11]. However, this approach is limited to molecules that can be modified in such a manner as to retain their therapeutic action. Sub-cellular targeted drug carrier systems that do not require the active molecule to be modified in any way can potentially be applied to a greater variety of drug molecule classes and may prove to be a powerful alternative if they can be developed. To this end it is interesting to note the work of Savic

*et al* who report that “nanocontainers” prepared from block copolymer micelles distribute, albeit randomly, to cell organelles [12]. The specificity of sub-cellular distribution does, however, leave much to be desired [13]. In our own earlier studies we have explored the use of amphiphilic derivatives with known and highly specific sub-cellular distribution to design organelle-specific pharmaceutical nanocarriers [14]. Our focus has been on the development of nanocarrier systems targeted to mitochondria.

Since the early 1990s, it has become increasingly evident that mitochondrial dysfunction contributes to a large variety of human disorders, ranging from neurodegenerative and neuromuscular diseases, obesity and diabetes to ischemia-reperfusion injury and cancer. Based on recent developments in pharmacological intervention aimed at mitochondria, “Mitochondrial Medicine” has emerged as a new field of biomedical research [15, 16]. The identification of mitochondrial drug targets in combination with the development of methods for selectively delivering biologically active molecules to the site of mitochondria could allow new therapies for the treatment of mitochondria-related diseases. However, the need for mitochondria-specific drug carrier systems able to selectively transport biologically active molecules to and into mitochondria within living human cells remains unmet [17, 18].

We have shown that it is possible to attach mitochondriotropic molecules to the surface of nanocarriers and that such nanocarrier systems can be loaded with drugs or DNA [1, 19]. The mitochondriotropic triphenylphosphonium (TPP) cation has been conjugated to various biologically active molecules to facilitate a selective accumulation of these molecules in mitochondria with the intent to probe, prevent or alleviate mitochondrial dysfunction [11, 20-23]. We conjugated TPP to a stearyl residue to give

stearyl triphenylphosphonium (STPP), an amphiphilic molecule that can be incorporated into lipid bi-layers [1]. STPP makes possible the preparation of liposome-based nanocarriers with TPP residues on the surface. These STPP liposomes specifically accumulate at mitochondria. We have recently shown that these mitochondria-specific liposomes can be used to deliver drugs to mitochondria and that such specific delivery to a sub-cellular site of action improves the action of paclitaxel and ceramide (drugs known to act on mitochondria) [2]. Our delivery approach can potentially be applied to other poorly water soluble drugs known to act on mitochondria as well.

Sclareol (labd-14-ene-8, 13-diol) (Fig.1) is a ditertiary alcohol, a member of the labdane type diterpenes. Sclareol was first isolated from the plant *salvia sclarea* (family Labiatae), and it is also found in many conifers. Sclareol exhibited antimicrobial activity and significant cytotoxic effects against leukemic cancer cell lines. It was found to induce cell cycle arrest and apoptosis, while down-regulating the expression of the proto-oncogene c-myc [24, 25].

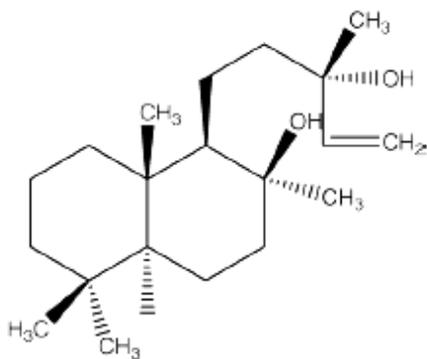


Fig. 1. Structure of sclareol.

Free sclareol exhibited growth inhibiting and cytotoxic activity against a variety of human cancer cell lines [3]. Despite its promising anticancer action *in vitro* the high systemic toxicity of sclareol, due most likely to its high lipophilicity, precluded the

evaluation of its anticancer effect *in vivo*. To overcome high toxicity and high lipophilicity of sclareol, it was incorporated in liposomes. Upon incorporation into liposomes, sclareol retained its growth-inhibiting activity against cancer cells but showed no cytotoxicity towards normal cells at concentrations as high as 100  $\mu\text{M}$  [3]. Most importantly, liposomal incorporation greatly improved pharmacological profile of the molecule *in vivo*. All mice injected with liposomal sclareol survived with no detectable manifestation of toxicity. Mice injected with the same dose of free sclareol exhibited severe behavioral changes and ataxia and died within 15 min to 3 days of injection. In a tumor reduction study, free sclareol showed no significant antitumor activity in HCT116 *in vivo*, but there was significant antitumor activity when sclareol incorporated liposomes were administered. Sclareol incorporated in liposomes resulted in almost 50% lower size of tumor than in control animals [4].

Sclareol was found to arrest cell cycle at G1 phase and induce apoptosis in human colon cancer HCT116 cells [4]. Activation of caspase-8 and caspase-9 in early stages followed by activation of caspase-3 and degradation of protein poly ADP-ribose polymerase (PARP) was observed. Activation of caspases showed that sclareol-induced apoptosis by activating both the mitochondrial pathway as well as the death receptor pathway [4]. Sclareol is thus a molecule that might potentially exert its apoptotic action by direct action on mitochondria. Incorporation of sclareol into our mitochondria-targeted liposomes could result in a dramatically improved cytotoxic action.

**METHODS: -****PREPARATION AND CHARACTERIZATION OF LIPOSOMAL FORMULATIONS:-****Preparation of liposomal formulations: -**

Liposomes were prepared by the thin-film hydration method [26]. The lipid film was prepared by adding egg-phosphatidylcholine (EPC) (Avanti polar lipids), dipalmitoylphosphatidylglycerol (DPPG) (Avanti polar lipids), Sclareol (Sigma Aldrich), STPP and 1, 2-Dioleoyl-3-Trimethylammonium-Propane (Chloride Salt) (DOTAP) (Avanti polar lipids) in different molar ratio as shown in Table I. The lipid film was dried under vacuum in a rotary evaporator. The lipid film was then hydrated with 0.292M sucrose (sucrose to lipid ratio 2.24 w/w) for 1 hr with occasional stirring. 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 (6 watts, 60 Sonic Dismembrator, Fisher scientific, USA). The resulting liposomal suspension was allowed to rest for 30 min and was centrifuged at 3000 rpm for 6 min at 25<sup>0</sup>C in order to separate the Small Unilamellar Vesicles (SUVs) from Titanium particles and from multi-lamellar vesicles (MLV's). Subsequently, the liposomal suspension was freeze-dried and stored at 4<sup>0</sup>C.

Freeze dried liposomal preparations were reconstituted to initial volume by adding HPLC-grade water. After addition of HPLC-grade water, preparations were allowed to rest for 15 min with occasional stirring before use.

Formulation	Lipid composition	Molar ratio
Empty non-targeted liposomes	EPC : DPPG	9 : 0.1
Sclareol incorporated in non-targeted liposomes	EPC : DPPG : Sclareol	9 : 0.1 : 5
Empty mitochondria targeted liposomes	EPC : DPPG : STPP	8.87 : 0.1 : 0.136
Sclareol incorporated in targeted liposomes	EPC : DPPG : STPP : Sclareol	8.87 : 0.1 : 0.136 : 5
Empty DOTAP liposomes	EPC : DPPG : DOTAP	8.87 : 0.1 : 0.136
Sclareol incorporated in DOTAP liposomes	EPC : DPPG : DOTAP : Sclareol	8.87 : 0.1 : 0.136 : 5

Table I: - Molar ratio of lipids for different liposomal formulations.

Characterization of liposomal formulations: -

Liposomal formulations were characterized by measuring Size distribution and Zeta potential, parameters that indicate a liposome's physical stability.

Size distribution of liposomal formulations: -

The size distribution of the liposomal formulation was determined using a Zeta Potential Analyzer (Zetaplus) instrument from Brookhaven Instruments Corporation. For each measurement, 100  $\mu$ l of the liposomal formulation was added to 2 ml of 1mM KCL, to obtain the proper intensity for measurement of the size distribution. The size distribution was obtained by collection of light scattering signal at an angle of 90 degrees.

Zeta-potential of liposomal preparations: -

The zeta potential of liposomal formulations was determined using the Zeta Potential Analyzer (Zetaplus) from Brookhaven Instruments Corporation. For each measurement, 100  $\mu$ l of the liposomal formulation was added into 2 ml 1mM KCL immediately after preparation as well as after reconstituting freeze-dried preparation.

### EVALUATION OF CYTOTOXICITY: -

The cytotoxicity of Sclareol was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega. It contains an MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). The MTS reagent is bio-reduced by cells into a colored formazan product whose absorbance can be measured at 490 nm. The absorbance measured at 490 nm is directly proportional to the number of living cells in culture. This assay was used to measure the cytotoxicity of sclareol incorporated in non-targeted liposomes on mammalian cells in culture.

COLO205 cells were grown in 96 well plates to 50-60% confluent. The cells were washed and treated with appropriate amount of sclareol incorporated into non-targeted liposomes and sclareol incorporated into targeted liposomes to achieve a 100 µM final concentration of sclareol per well. The cells were incubated for 24 hrs. Following incubation, the cells were washed and 10 µl MTS reagent per well was added. After incubation for 1 hour, the resultant absorbance was measured at 490 nm. % cell death was calculated using the following formula:-

$$\% \text{ cell death} = (\text{ABS}_c - \text{ABS}_s) * 100 / \text{ABS}_c$$

Where,  $\text{ABS}_c$  = Absorbance of control (untreated) cells  
 $\text{ABS}_s$  = Absorbance of cells treated with sclareol incorporated in non-targeted liposomes or sclareol incorporated in targeted liposomes.

## EVALUATION OF APOPTOTIC ACTIVITY: -

### Apoptosis: -

Defective apoptosis represents a major causative factor in the development and progression of cancer. Apoptosis is one of the main types of programmed cell death and can be induced by number of factors including DNA damage and growth factor deprivation. Apoptosis is characterized by cell shrinkage, blebbing of plasma membrane, chromatin condensation and DNA fragmentation [27]. The central component of this process is a proteolytic system involving a family of proteases called caspases. Caspases, also known as cysteine aspartate-specific proteases, are a family of intracellular proteins involved in the initiation and execution of apoptosis. Initiator caspases are able to activate effector caspases or amplify caspase cascades by increased activation of initiator caspases [28]. Then the effector caspases cleave intracellular substrates, culminating in cell death [28, 29]. Apoptosis is mainly transduced by two cascades called the extrinsic pathway and the intrinsic pathway. Both pathways involve the activation of the caspase activation cascade. The extrinsic pathway, also known as the death receptor mediated pathway, is triggered at the cell surface and involves the activation of caspase-8. Caspase 8 activation occurs through activation of the death receptors FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [30]. Caspase 8 activates caspase 3 with or without the activation of caspase 9 depending upon the cellular injury [31]. The intrinsic pathway also called the mitochondria-dependent pathway is initiated with the release of cytochrome c from the mitochondrial intermembrane space [32]. The released Cytochrome c binds to Apaf-1 in the cytosol resulting in the activation of the initiator caspase, caspase-9 [33]. Caspase-9 activates caspase-3 leading to nucleosomal DNA

fragmentation [34]. Subsequently caspase-8 or -9 can activate caspase-3, which in turn targets and degrades specific and vital cellular proteins, ultimately resulting in nuclear condensation and DNA degradation that are the final hallmarks of apoptotic cell death.

The proapoptotic activity of sclareol was measured using a nuclear morphology assay, fluorometric caspase activity assay and detection of apoptotic cells using a flow cytometry method.

#### Nuclear Morphology Assay: -

The blue fluorescent Hoechst dye (33342 Molecular Probes from Invitrogen) stains nucleic acid in cells. Hoechst dye was used to study nuclear morphology in cells treated with sclareol.

Human colon carcinoma, COLO205 cells were grown on glass coverslips in six well culture plates till 75% confluent. Cells were then treated in duplicate for 72 hrs with sclareol incorporated in non-targeted liposomes to achieve 100  $\mu\text{M}$  final concentration of sclareol. After incubation, the cells were stained with Hoechst for 5min. The liquid contents of the wells were collected and centrifuged to recover floating cells. The coverslips were washed with fresh medium. The cell pellet recovered from the well contents was re-suspended in 100  $\mu\text{l}$  of mounting medium. Ten  $\mu\text{l}$  of the suspension was placed on a glass slide and the corresponding coverslip was placed on top. The slides thus prepared were examined under a UV fluorescence microscope.

Caspase assay: -

Caspase 8 activity was measured using Caspase-8/FLICE Fluorometric Assay Kit from Biovision and caspase 9 activity was measured using Caspase 9 Fluorometric assay kit from Biovision.

Caspases, the family of proteases, recognize and cleave specific peptide sequences [35]. Caspase 8 recognizes the sequence IETD. The assay is based on detection of cleavage of substrate IETD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). IETD-AFC emits blue light ( $\lambda_{\text{max}} = 400 \text{ nm}$ ); upon cleavage of the substrate by caspase 8, free AFC emits a yellow-green fluorescence ( $\lambda_{\text{max}} = 505 \text{ nm}$ ), which can be quantified using a fluorometer or a fluorescence microtiter plate reader. Similarly, Caspase 9 recognizes the sequence LEHD. The assay is based on detection of cleavage of substrate LEHD-AFC. LEHD-AFC emits blue light ( $\lambda_{\text{max}} = 400 \text{ nm}$ ); upon cleavage of the substrate by caspase-9 or related caspases, free AFC emits a yellow-green fluorescence ( $\lambda_{\text{max}} = 505 \text{ nm}$ ), which can be quantified using a fluorometer or fluorescence microtiter plate reader.

Human colon carcinoma, COLO205 were grown in T75 culture flask till 75% confluent. Cells were then treated for 24 hrs with sclareol incorporated in non-targeted liposomes and sclareol incorporated into targeted liposomes to achieve 100  $\mu\text{M}$  final concentration of sclareol per flask. After incubation, cells were collected and centrifuged to get cell pellets. Cell pellets were resuspended in PBS. The cells were then counted using a Neubauer Slide. Aliquots containing 1 million cells were then used to measure caspase-8 and caspase-9 activity according to the instructions supplied with the respective kits. Caspase activity was determined for treated as well as untreated cells.

Results are expressed as the percent increase in caspase activity in treated samples over untreated control.

Annexin V binding assay: -

Vybrant Apoptosis Assay kit #3 from invitrogen was used to detect cells under going apoptosis. In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, annexin V, is a 35-36 kD  $\text{Ca}^{+2}$ -dependent phospholipid binding protein that has a high affinity for PS. Annexin-V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet.

The vibrant Apoptosis Assay Kit #3 contains recombinant annexin V conjugated to fluorescein (FITC – annexin V) as well as red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to nucleic acids in the cell. After staining a cell population with FITC annexin V and PI, apoptotic cells show green fluorescence, dead cells show red and green fluorescence and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation.

Human colon carcinoma, COLO205 were grown in T25 culture flask till 75% confluent. Cells were then treated for 8 hrs with sclareol incorporated in non-targeted liposomes and sclareol incorporated into targeted liposomes to achieve 100  $\mu\text{M}$  final

concentration of sclareol per flask. After incubation, cells were collected and centrifuged to get cell pellets. Cell pellets were resuspended in PBS. The cells were then counted using a Neubauer Slide. Aliquots containing 0.2 million cells were then used to measure apoptotic cells by flow cytometry according to manufacturer's protocol. Results are expressed as the percent increase in cells under going apoptosis in treated cells over untreated control.

**STATISTICAL ANALYSIS: -**

Differences among two treatment groups were statistically analyzed using a two tailed t test. A statistically significant difference was reported with p value. Data are reported as the mean  $\pm$  standard deviation. All statistics were done using Microsoft Office Excel.

## RESULTS AND DISCUSSION: -

### PREPARATION AND CHARACTERIZATION OF LIPOSOMAL FORMULATIONS:-

Sclareol was incorporated into non-targeted liposomes and mitochondria targeted liposomes at final compositions shown in preparation of liposomal formulations methods section. The liposomal formulations were freeze dried and after rehydration, the size distribution and zeta potential were measured.

The results from the size distribution and zeta potential study are summarized in table II. We can see that size distribution for liposomal formulations is consistent and shows preparation of stable formulations.

	Size distribution		Zeta potential	
	Before freeze drying	After freeze drying	Before freeze drying	After freeze drying
Empty non-targeted liposomes	64.20 ± 5.46	73.33 ± 8.19	-31.40 ± 2.30	-31.72 ± 3.28
Sclareol incorporated in non-targeted liposomes	77.05 ± 11.24	88.25 ± 3.46	-35.1 ± 12.50	-38.21 ± 4.32
Empty targeted liposomes	72.73 ± 14.64	107.15 ± 2.75	11.27 ± 3.95	12.44 ± 2.20
Sclareol incorporated in targeted liposomes	76.66 ± 4.71	105.35 ± 17.18	6.63 ± 4.91	9.85 ± 3.83
Empty DOTAP liposomes	69.73 ± 6.90	103.76 ± 3.85	7.69 ± 4.54	9.09 ± 1.60
Sclareol incorporated in DOTAP liposomes	67.8 ± 7.16	89.1 ± 9.62	0.13 ± 5.86	3.03 ± 1.08

Table II: - Size distribution and zeta potential for different liposomal formulations before and after freeze drying. Data are reported as mean ± standard deviation from three separate readings.

We surface modified liposomes with STPP cations to target mitochondria. As STPP cations are exposed on the outer surface of liposomes, zeta potential for sclareol incorporated into liposomes surface modified with STPP bear overall positive charges compared to negative charge of sclareol incorporated into non-targeted liposomes. We

used empty DOTAP liposomes and sclareol incorporated into DOTAP liposomes as one of the controls to compare the effect of change in zeta potential of liposomes after surface modification with targeting ligands. With these stable preparations, we carried out cytotoxicity assays and proapoptotic assays.

#### EVALUATION OF CYTOTOXICITY: -

The cytotoxicity of sclareol incorporated into non-targeted liposomes and sclareol incorporated into targeted liposomes was investigated in COLO205 cells. The data showed a statistically significant increase in cytotoxic activity of sclareol in cells treated with sclareol incorporated into targeted liposomes compared to sclareol incorporated into non-targeted liposomes. As can be seen in figure 2, at 100  $\mu$ M sclareol concentration, sclareol incorporated into targeted liposomes killed approximately 60% of COLO205 cells, while the sclareol incorporated into non-targeted liposomes killed only 40% of COLO205 cells.

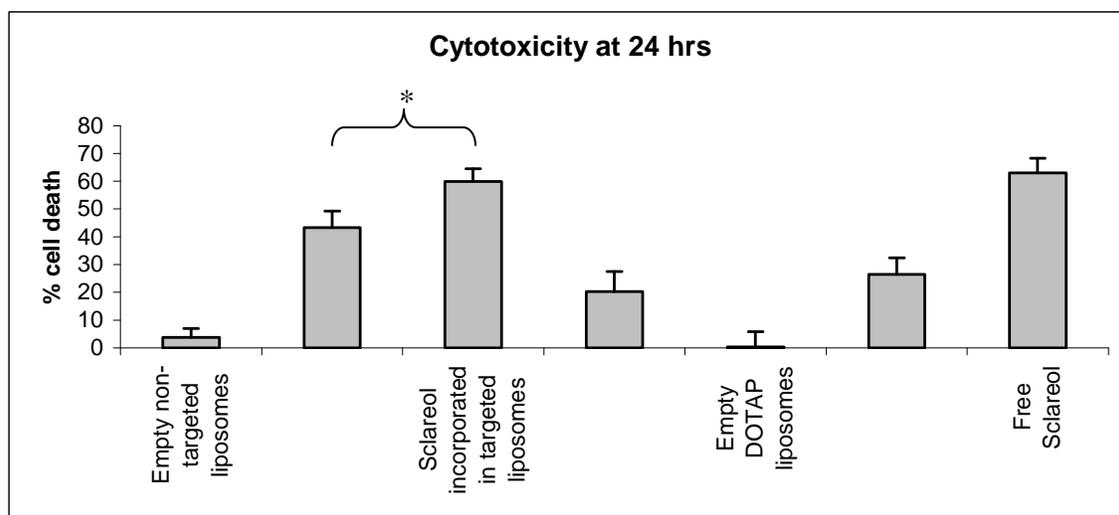


Figure 2. Evaluation of cytotoxicity of sclareol at 24 hrs in COLO205. \*,  $p < 0.05$  (n=3).

## EVALUATION OF APOPTOTIC ACTIVITY; -

### Nuclear morphology assay: -

We examined the nuclear morphology of COLO205 cells treated with sclareol incorporated into non-targeted liposomes to establish a time line to detect the early apoptotic events such as caspase activation and detection of apoptotic cells by flow cytometry. As seen in figure 3, we observed approximately 20% of cells treated with sclareol incorporated into non-targeted liposomes showed nuclei condensation at 48 hrs which is a late apoptotic event. Using this data, we determined activation of caspase-8 and caspase-9 at 24 hrs which is an early apoptotic event.

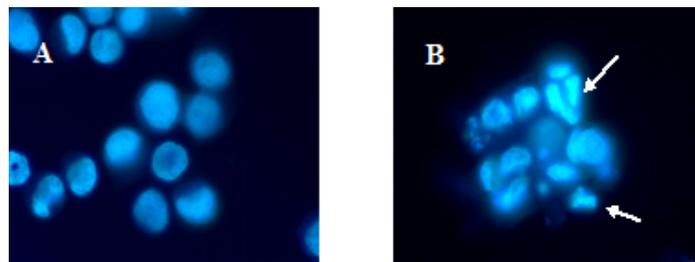


Figure 3: Nuclear morphology assay in COLO205 at 48 hrs. Nuclear morphology of COLO205 cells treated with A) Negative control B) Sclareol incorporated into non-targeted liposomes at final concentration of 100  $\mu$ M. Arrows in figure indicate apoptotic nuclei.

Caspase assay: -

Caspase-8 and caspase-9 activity were measured using commercially available fluorometric caspase activity assay kit from Biovision. Activation of caspase-9 represents apoptosis induction via mitochondrial pathway and activation of caspase-8 represents apoptosis inductions via death receptor pathway.

COLO205 cells were incubated with 100  $\mu$ M of Sclareol for 24 hrs. Caspase-9 and caspase-8 activity were measured. As seen in figure 4 (B), Caspase-9 activity in cells treated with sclareol incorporated into mitochondria targeted liposomes is almost three fold than sclareol incorporated into non-targeted liposomes. These results indicate change in potency due to targeting of sclareol to mitochondria and increase in proapoptotic activity using sub-cellular targeted liposomes.

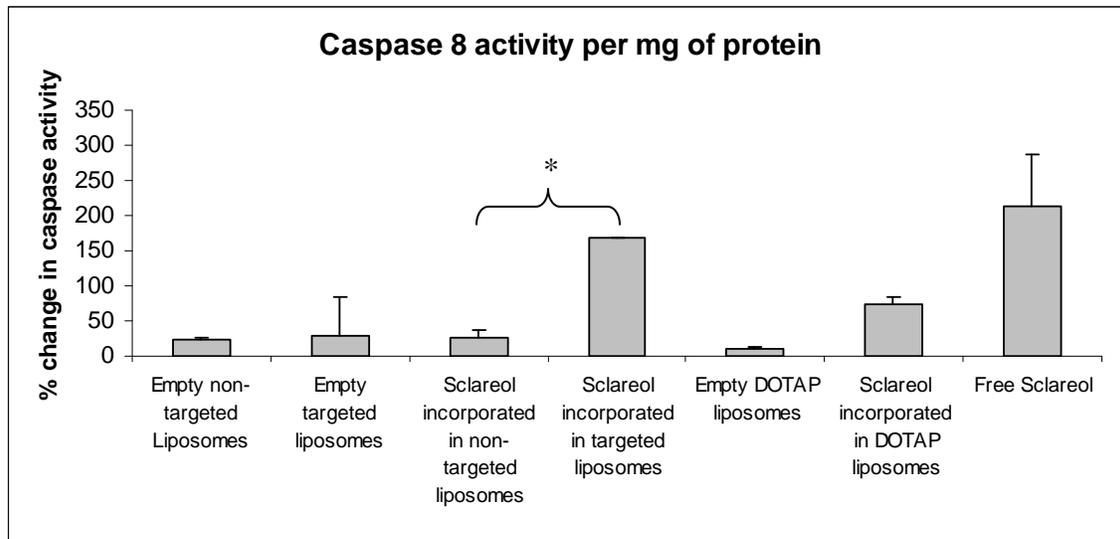


Figure 4 (A): - Evaluation of caspase activity. Figure shows activation of caspase-8 at 24 hrs in COLO205 cells treated with sclareol incorporated into non-targeted liposomes and sclareol incorporated into targeted liposomes. \*  $p < 0.05$  (n=3).

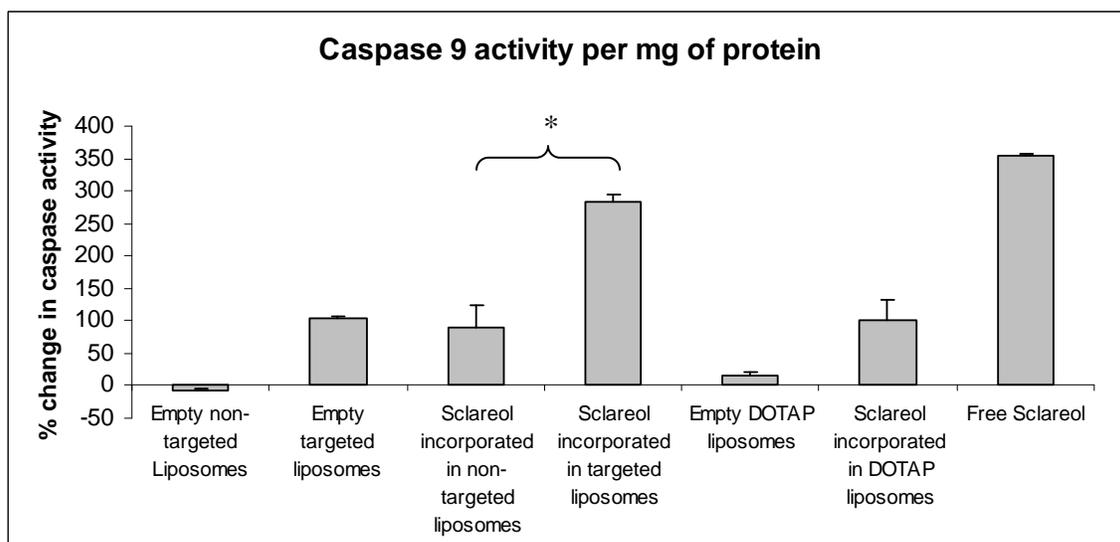


Figure 4 (B): -Evaluation of caspase activity. Figure shows activation of caspase-9 at 24 hrs in COLO205 cells treated with sclareol incorporated into non-targeted liposomes and sclareol incorporated into targeted liposomes. \*  $p < 0.05$  (n=3).

### Annexin V binding assay:-

Apoptotic cells were counted by Vybrant® Apoptosis assay kit # 3 using flow cytometry. As seen in figure 5, number of cells undergoing apoptosis is almost three fold increased when treated with sclareol incorporated into targeted liposomes compared to sclareol incorporated into non-targeted liposomes.

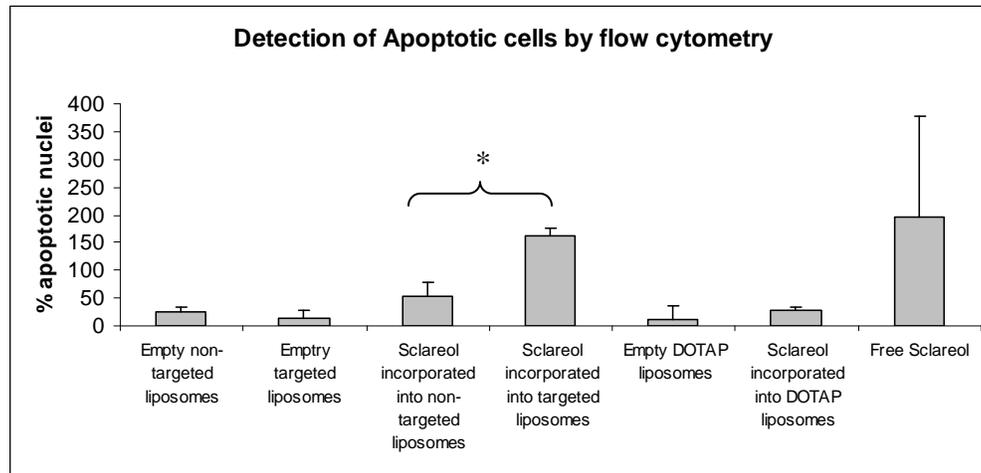


Figure 5: - Detection of apoptotic cells by flow cytometry in COLO205 cells at 8 hrs.  
\*,  $p < 0.05$  (n=3).

**SUMMARY AND CONCLUSION: -**

A major challenge in treating cancer cells is the lack of selectivity of the currently available cytotoxicity agents. Another major hurdle to treat cancer successfully is sub-cellular targeting of drug molecules. So, to efficiently and selectively eradicate carcinoma cells, the drug molecules not only need to be delivered to the tumor cells, but also to the particular target inside the cells. This approach becomes increasingly feasible as the particular mechanism of action, i.e. the molecular targets of anti-cancer drugs are being recognized. Transporting the cytotoxic drug to its intracellular target would significantly increase the sub-cellular bioavailability of any drug acting inside a cell.

We used STPP to surface modify liposomes and render them tumor cell and mitochondria-specific delivery system for established and experimental anticancer drugs known to trigger apoptosis by acting on mitochondria. We have demonstrated potential of such a system to deliver ceramide to mitochondria and this sub-cellular targeting of ceramide resulted in its increased potency. The purpose of this study was to demonstrate broad applicability of such a system to increase potency of drugs known to act on mitochondria by delivering them to its sub-cellular site of action, mitochondria. We chose sclareol as a model drug as it induces apoptosis in cancer cells by acting on mitochondria. Our hypothesis was that sclareol incorporated in mitochondria-targeted liposomes would exhibit improved pro-apoptotic and cytotoxic action on cancer cells *in vitro*. To test our hypothesis, we incorporated sclareol into non-targeted liposomes and targeted liposomes. Characterization of these liposomal formulations indicated that the formulations were stable. These formulations were chosen for further analysis *in vitro*.

According to our rationale, Sub-cellular targeting of drug molecules should result in an increased efficacy of drugs. *In vitro* experimental data showed increased cytotoxic activity and proapoptotic activity of sclareol when incorporated into targeted liposomes compared to non-targeted liposomes. Results of this project strongly support the initial hypothesis that sclareol incorporated in mitochondria targeted liposomes will exhibit improved pro-apoptotic and cytotoxic action on cancer cells *in vitro*. By doing so, we demonstrated broad applicability of mitochondria-targeted liposomes in delivering therapeutic molecules with diverse physicochemical properties to their sub-cellular site of action, mitochondria.

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