ISSN: 2230-9926

*Available online at http://www.journalijdr.com*

IJDR

*International Journal of Development Research*
*Vol. 4, Issue, 3, pp. 753-756, March, 2014*

*International Journal of*
**DEVELOPMENT RESEARCH**

## *Full Length Research Article*

# THE APOPTOTIC EFFECT OF THE MICROEMULSION FORMULATION OF SIMVASTATIN/ CREMOPHOR EL/ TRANSCUTOL/ CAPTEX355/WATER IN A549 NON-SMALL CELL LUNG CANCER CELLS

**Mayson H. Alkhatib* and Sahar S. AL-Merabi**

Department of Biochemistry, College of Science, King Abdulaziz University, P.O. Box 42801, Jeddah 21551, Saudi Arabia

**ARTICLE INFO**

***Article History:***
Received 22nd December, 2013
Received in revised form
19th January, 2014
Accepted 12th February, 2014
Published online 25th March, 2014

***Key words:***
Microemulsions;
Antitumor activity;
Cytotoxicity;
Light microscopy.

**ABSTRACT**

The antiproliferative effect of the lipid lowering agent, simvastatin, has recently grabbed the attention of the pharmaceutical industries. The apoptotic effect of simvastatin, encapsulated in a microemulsion formula composed of Cremophor EL, Transcutol, Captex 355 and water, was assessed in the A549 non-small cell lung cancer cells. The cytotoxicity of and Zocor, blank microemulsion (BM), the freshly prepared microemulsion formula (FDM) and the old FDM (ODM), which was kept for two weeks, were screened using sulphorhodamine B (SRB) assay, ApopNexin FITC apoptosis detection kit and light microscopy. It has been found that FDM and ODM caused cell mortalities more than Zocor by 2.2 and 2.4 folds. The signs of apoptosis, such as chromatid condensation, membrane blebbing and increased intracellular spaces between the cells, were obviously seen in the A549 cells treated with FDM and ODM. Our findings demonstrate that the microemulsion improved the antitumor effect of simvastatin against A549 cells.

*Copyright © 2014 Mayson H. Alkhatib and Sahar S. AL-Merabi. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.*

## INTRODUCTION

The oral-based drug delivery system is the most common way to deliver drugs into the bloodstream. In general, water-soluble drugs can diffuse freely and easily into the gastrointestinal tract with high bioavailability. However, most of the drugs discovered nowadays with the advances in biotechnology and pharmaceutical technology are oil-soluble which pose serious problems in diffusion and absorption within the body due to their poor solubility (Spernath and Aserin, 2006). One way to deliver oil-soluble drugs is to incorporate them into an inert lipid vehicle, such as microemulsions, and liposomes. This could lead to increased solubilization with concomitant modification of their pharmacokinetic profiles and improvement in the therapeutic efficacy (Schwendener and Schott, 1996; Burcham et al., 1997). Microemulsions proved to be good potential drug delivery vehicles for poorly water-soluble drugs. They are homogeneous, transparent, thermodynamically stable dispersions of water and oil stabilized by a surface active agent or surfactant, usually in combination with a co-surfactant like alcohol (Hoar and Schulman, 1943; Paul and Moulik, 1997). Surfactant molecules, such as fatty acids, exhibit a very peculiar behavior because they contain both polar and non-polar group. They tend to adsorb at the interface of the oil and water mixture, where they can fulfill their dual affinity with hydrophilic groups located in the aqueous phase and hydrophobic groups in oil. Additionally, they reduce the mismatch with solvent through a specific kind of aggregation process known as micellization. Depending on the fractions of oil and water, microemulsions can be classified as oil-in-water microemulsion (o/w μe), water-in-oil microemulsion (w/o μe), or bicontinuous systems (Alkhatib et al., 2012). In particular, o/w μe system is produced when oil molecules are dispersed in the continuous phase of water. Accordingly, hydrophobic drugs that are soluble in oil might be dispersed inside the water. On the other hand, w/o μe system is formed if water molecules are solubilized in the continuous oil phase. These systems are usually applied for the hydrophilic drugs if used for transdermal deliveries. At approximate equal fractions of both oil and water, bicontinuous systems is produced, which hold a great potential for biochemical separations, especially, proteins. These versatile microemulsion systems, that produce small dispersed droplets with diameters in the range of 10 -140 nm, are currently of great technological and scientific interest

***Corresponding author: Mayson H. Alkhatib***
*Department of Biochemistry, College of Science, King Abdulaziz University, P.O. Box 42801, Jeddah 21551, Saudi Arabia*

to the researchers. They provide protection against oxidation, enzymatic hydrolysis and improve the solubilization of lipophilic drugs which enhance their bioavailability. In addition to oral and intravenous delivery, they are amenable for sustained and targeted delivery through ophthalmic, dental, pulmonary, vaginal and topical routes (He et al., 2010). One of the very hydrophobic drugs that possess a low systemic bioavailability coupled with extensive first pass hepatic metabolism is simvastatin, which was involved in microemulsions for solubility enhancement (Schachter, 2005; Mandal et al., 2010). Some studies approved delivery of simvastatin by using nanoparticles from microemulsions (Margulis-Goshen and Magdassi, 2009). Simvastatin was formulated using a 1:1 (*V/V*) mixture of diesters of caprylic/capric acids and polyglycolyzed glycerides with varying concentrations of polyoxy castor oil and C8/C10 mono-/diglycerides (Patil et al., 2007). Another study used diethylene glycol monoethyl ether, polyglycolysed glycerides and cremophor and C-18 solid-phase extraction cartridges to formulate simvastatin microemulsions (Kang et al., 2004). Simvastatin is widely used to treat hypercholesterolemia (Jun et al., 2007). It is a member of the statin class of pharmaceuticals that have been used extensively in the past decade and theiruse is likely to continue rising. However, recent researches have showed controversial results regarding their impact on cancer. Early review of studies in rodents and at least two clinical trials of pravastatin showed that statins may have carcinogenic properties (Schwendener and Schott, 1996;He et al., 2010; Paul and Moulik, 1997). On the other hand, a growing body of mechanistic studies suggested that statins may have chemopreventive potential against cancer (Schachter, 2005). Some studies showed that statins seem to induce apoptosis and reduce cell invasiveness in various cell lines, including mammary carcinoma (Jun et al., 2007).

## MATERIALS

The human cell lines of A549 non-small cell lung cancer and all of the tissue culture reagents were obtained from the Tissue Culture Bank at King Fahd Medical Research Center, Jeddah, KSA. Simvastatin was provided by Pharma Jamjoom, Jeddah, KSA. Polyoxyl 35 castor oil (Cremophor EL), Transcutol, diesters of caprylic/ capric acids (Captex 355) were purchased from Sigma-Aldrich Chemical Co, St Louis, MO, USA. Commercial simvastatin (Zocor) was obtained from King Abdulaziz University Hospital, Jeddah, KSA. Distilled water was purified using a water purification system from Bibbysterilin ltd, UK. ApopNexin FITC Apoptosis Detection Kit was purchased from Millipore, MA, USA.

## METHODS

### Preparation of the micro emulsion formulation

The microemulsion formula was prepared by mixing the weight fractions percentages of 24.6% of Cremophor EL, 12.3% of Transcutol, 29% of Captex 355 and 34 % of water. A 1mg/ml of simvastatin was dissolved directly in the microemulsion formula.

### Cell culture

A549 cells were grown in a tissue culture flask (75 $cm^3$) containing 10 ml of modified eagle medium (MEM) media supplemented with 10% (v/v) heat inactivated fetal calf serum at 37°C in a 5% $CO_2$ incubator. The spent Medium was discarded from the tissue culture flask and changed at 48 h intervals. Cells were fed until they reached confluence. Confluent cells were collected by trypsinization, washed and passaged every 3 days. The cells, used for the experiments, were between passages 7 and 11. They were dissociated with 2 ml of trypsin (0.15M) added to the tissue culture flask, left for few seconds and then discarded. This process was repeated two times with extending the second time to three min. The experimental cells were incubated in MEM media (10%) for 24 h in a 5% $CO_2$ incubator at 37°C.

### Sulphorhodamine B (SRB) assay for cytotoxicity assessment

The anti-proliferative assay, sulphorhodamine B (SRB) assay, was performed according to method of Skehan et al. (1990). Cultured cells of A549 were counted using a hemocytometer and seeded at a density of 1 $x10^5$ cells per 100 µl culture media per well of 96-well flat-bottomed microliter plates containing 0.1 ml of growth media per well. Cells were incubated with 0.1 ml of 25 µM, 50 µM and 100µM concentration of blank microemulsions (BM), fresh (FDM) and old (aged for 2 weeks) simvastatin loaded-microemulsion (ODM) and Zocor solution, solubilized in the media. Triplicate wells were prepared for each individual concentration and re-incubated for additional 48 h at 37°C in a 5% $CO_2$ incubator. Untreated cells were used as control. The numbers of living cells were assayed by measuring the color intensity using enzyme-linked immunosorbent assay, ELISA, reader at a wavelength of 490 nm. The ratios of vital cells to dead cells were determined to evaluate the cytotoxicity of BM, FDM, ODM and Zocor each against A549 cells. The cytotoxicity effect was determined by measuring the percentages of cell viability using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance af the sample}}{\text{Absorbance of the control}} \times 100 ,$$

where the absorbance of the sample and the absorbance of the control were defined as the absorbance of the treated and untreated cells, respectively, measured at 490nm. Characterization of cell morphology using light microscopy
Cultured A549 cells were counted and plated at a density of 1 $x10^5$ cells per well into 96-well, flat-bottomed microliter plates, containing 0.1 ml of growth medium per well. Cells were incubated with 0.1 ml of 25 µM, 50 µM and 100 µM of FDM, ODM and Zocor. Cells were washed with 4% formaldehyde and stained with 10% Coomassie blue and left to dry at room temperature. Excess stain was washed with phosphate buffer solution three times. Morphological changes were observed by a phase contrast inverted microscope (TH4-200, Olympus optical Co-ltd, Japan).

### Detection of apoptosis by ApopNexin FITC assay

The signs of apoptosis induced by 50 µM of FDM, ODM and Zocor were inspected by an ApopNexin FITC Apoptosis Detection kit (Millipore, Lot. No. 2053919, Billerica, MA, USA). This kit uses a staining protocol in which the apoptotic cells are stained with annexin V conjugated with fluorescein isothiocyanate (FITC) (gives green fluorescence) which stains phosphatidylserine (PS). Cultured A549 cells were plated into

24-well plates ($2\times10^4$ cells per well) and incubated for 24 h. The formulations of 50 µM of BM, FDM, ODM and Zocor were introduced to the cells and incubated for another 48 h and were treated according to the protocol description of ApopNexin FITC Apoptosis Detection kit.

### Statistical analyses

All values were expressed as mean ± standard deviation ($\bar{X} \pm SD$)as each experiment was performed in triplicate. Statistical analyses were performed with the one-way analysis of variance (ANOVA) test and independent sample *t*-test using the MegaStat. The significance difference was considered when $P < 0.05$.

## RESULTS

### SRB assay for cytotoxicity assessment

As illustrated in Table 1, the cell viability percentages of FDM and ODM slightly changed as the concentration increased and gave approximately similar percentages of cell viability that were less than Zocor at 50µM by 2.2 and 2.4 fold, respectively. BM at all selected concentrations was having weak effect on the A549 cells relative to other two formulas.

**Table 1. The cytotoxic effect of the different concentrations of microemulsion formulations subjected unto A549 cells. The percentages of cell viability were expressed as mean ± SD $(\bar{X} \pm SD), n = 3$**

| Formulations | Concentration (µM) | % Cell Viability |
|---|---|---|
| Fresh D-ME | 25 | 14.71 ± 1.68 |
| | 50 | 18.63 ± 1.23 |
| | 100 | 16.19 ± 2.12 |
| Old D-ME | 25 | 16.33 ± 1.26 |
| | 50 | 20.18 ± 5.83 |
| | 100 | 18.40 ± 4.17 |
| Blank-ME | 25 | 86.85 ± 1.93 |
| | 50 | 77.52 ± 3.48 |
| | 100 | 68.63 ± 1.60 |
| Zocor | 50 | 44.57 ± 8.43 |

### Characterization of cell morphology using light microscope

In order to understand the mechanism of cell death, which could be either necrosis or apoptosis, the cells morphologies were characterized using light microscopy. The signs of apoptosis, revealed by light microscopy images, include cell shrinkage, chromatin condensation, membrane blebbing, nuclear fragmentation and apoptotic body formation. The A549 cancer cells were more sensitive to the FDM and ODM than Zocor as the cell numbers were more reduced and showed more condensed chromatin with an increase in the number of apoptotic bodies (Figure 1).

### Detection of apoptosis by Apop Nexin FITC assay

As exhibited in Figure 2, the untreated cells did not stain positively with neither dyes, which indicates the viability of cells, while all the cells treated with the FDM and ODM were stained positively fluorescent green with annexin-V-FITC but not with PI, which implies the presence of apoptosis with no detectable necrotic effect due to the externalization of phosphatidylserine caused by the cell surface outbreak. Cells treated with Zocor were less stained indicating less apoptotic effect.

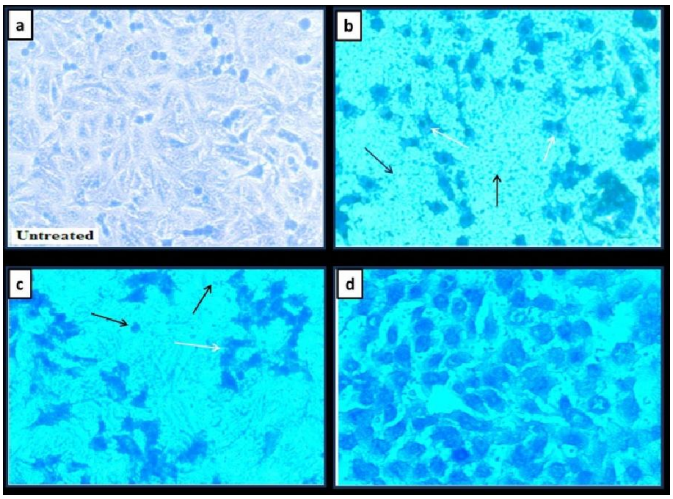


**Figure 1. Light microscopic images of (a) untreated and treated A549 cells with 50 µM of (b) FDM, (c) ODM and (d) Zocor. Images were magnified at 10x. *Black arrows* represent the apoptotic bodies while *white arrows* represent the membrane blebbing**

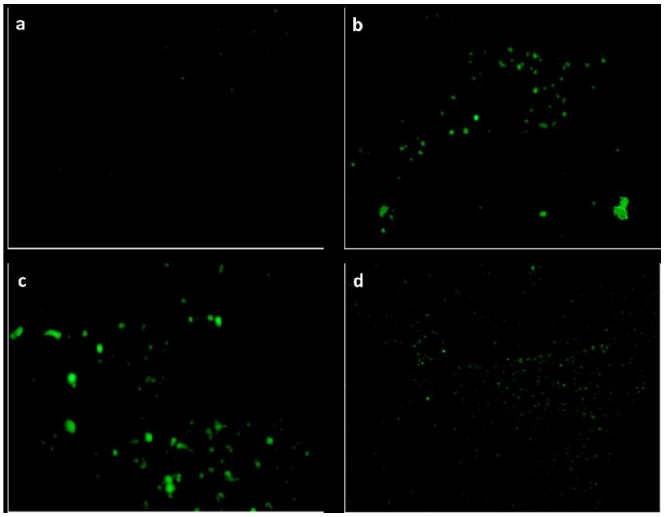


**Figure 2. Fluorescence microscopy images, labeled with ApopNexin FITC, showing the apoptosis of A549 cells when (a) untreated, and treated with 50 µM of (b) FDM, (c) ODM and (d) Zocor. Images were magnified at 20x**

## DISCUSSION

Simvastatin, a HMG CoA reductase inhibitor, is widely used to lower blood cholesterol level. However, this drug is associated with certain drawbacks like low oral bioavailability (5%) due to extensive hepatic first-pass metabolism (Brunton et al., 2006) and susceptibility to hydrolytic degradation in the gastrointestinal (GI) tract (Shah et al., 2011). Cytochrome P450 3A4 system is responsible for its degradation in liver. To overcome hepatic first-pass metabolism and to enhance bioavailability, intestinal lymphatic transport of the drug can be exploited and that can be possibly done by using lipids as a drug carrier (Nishioka and Yoshino, 2001). Lipids can enhance lymph formation and simultaneously promote lymph flow rate. Transport of drugs through the intestinal lymphatics via the thoracic lymph duct to the systemic circulation at the junction of the jugular and left subclavian vein avoids presystemic hepatic metabolism and therefore enhances bioavailability (Suresh et al., 2007). Microemulsion was selected for this study since there are no studies that determine

the antitumor effect of the simvastatin loaded-microemulsion. Most of the studies focus on the role of microemulsion in developing the effect of simvastatin in reducing the cholesterol in the blood. For instance, a recent study has found out that the microemulsion improved the solubility and *in vitro* release of simvastatin compared to the commercial tablet (Srinivasi et al., 2011; Srinivasi and Sagar, 2012). The dissolution rate and intestinal permeation of simvastatin increased significantly when it was delivered in a microemulsion that consisted of oleic acid, Cremophore RH 40, Transcutol, and water. Another study has shown an enhanced bioavailability of simvastatin when formulated in a self-micro-emulsifying drug delivery system, which consisted of mixtures of diesters of caprylic/capric acid, polyglycolyzed glycerides, polyoxy castor oil and C8/C10 mono-/diglycerides (Patil et al., 2007). Furthermore, atorvastatin, which was solubilized in a microemulsion formulation that composed of Labrafil 1944CS, Cremophor RH 40, ethanol and distilled water, was having improved gastrointestinal absorption in rats (Mandal et al., 2010). In this study, it has been found that the anticancer activity of FDM and ODM did not differ indicating the stability of the microemulsion formula for at least two weeks. In addition, FDM and ODM cytotoxicities were double the toxicity of Zocor. In general, statins have a great potential as anticancer agent with a high therapeutic index (Jakobisiak and Golab, 2003) although the clinical findings have drawn controversial conclusions (Emberson et al., 2012).

## CONCLUSIONS

In this study, the antitumor activity of simvastatin loaded in a microemulsion formula was double the effect of Zocor against A549 non-small cell lung cancer cells. The cell toxicity and the apoptotic effect were improved. It is recommended to implement more *in vivo* research studies to clarify the apoptotic of the drug formula.

## ACKNOWLEDGEMENTS

The authors wish to express a sincere thanks and appreciating to King Abdulaziz City for Science and Technology for its financial support to the research project designated by number (P-S-11-611), King Abdulaziz University Hospital for providing cell cultures and King Fahd Medical Research Center, Jeddah, KSA, for technical support.

## REFERENCES

Alkhatib, M., Albishi, H., Mahassni, S. 2012. Impact of Nanoparticles on Cancer Therapy. *Trop. J. Pharm. Res*., 11(6): 1001-1011.

Brunton, L., Lazo, J. and Parker, K. 2006. Goodman, and Gilman's The Pharmacological Basis of Therapeutics, Edited by: L.L. Brunton, J.S. Lazo and K.L. Parker, New York, McGraw-Hill.

Burcham, D., Maurin, M., Hausner, E. and Huang, S. 1997. Improved oral bioavailability of hypocholesterolemic DMP in dogs following dosing in oil and glycol solutions. Biopharm. *Drug Dispos*., 18:737-42.

Emberson, JR., Kearney, PM., Blackwell, L., Newman, C., Reith, C., Bhala, N., Holland, L., Peto, R., Keech, A., Collins, R., Simes, J., Baigent, C. 2012. Lack of effect of lowering LDL cholesterol on cancer: meta-analysis of individual data from 175,000 people in 27 randomised trials of statin therapy. PloS One; 7: e29849.

He, C., He, Z.andGao, J. 2010. Microemulsions as drug delivery systems to improve the solubility and the bioavailability of poorly water-soluble drugs. *Expert Opin. Drug Deliv*., 7:445-60.

Hoar, T. and Schulman, J. 1943. Transparent water in oil dispersions: the oleopathichydromicelle. *Nature*, 152: 102-107.

Jakobisiak, M., Golab, J. 2003. Potential antitumor effects of statins (Review). *Int. J. Oncol*., 23: 1055–1069.

Jun, S., Kim, M., Kim, J., Lee, S., Woo, J. and Hwang, S. 2007. Preparation and characterization of simvastatin/ hydroxypropyl-b-cyclodextrin inclusion complex using supercritical antisolvent (SAS) process. *Eur. J. Pharm. Biopharm*., 66: 413–421.

Kang, B., Lee, J., Chon, S., Jeong, S., Yuk, S., Khang, G., Lee, H. and Cho, S. 2004. Development of self-micro emulsifying drug delivery systems (SMEDDS) for oral bioavailability enhancement of simvastatin in beagle dogs. *Int. J. Pharm*., 274: 65-73.

Mandal, S., Mandal, S. and Sawant, K. 2010. Design and development of microemulsion drug delivery system of atorvastatin and study its intestinal permeability in rats. *International Journal of Drug Delivery*, 2: 69-75.

Margulis-Goshen, K. and Magdassi, S. 2009. Formation of simvastatin nanoparticles from microemulsion. Nanomedicine., 5:274–281.

Nishioka, Y. and Yoshino, H. 2001. Lymphatic targeting with nanoparticulate system. *Adv. Drug Deliv. Rev*., 47:55–64.

Patil, P., Patil, V. and Paradkar, A. 2007. Formulation of a self-emulsifying system for oral delivery of simvastatin: In vitro and in vivo evaluation. *Acta Pharm*., 57:111-122.

Paul, B. and Moulik, S. 1997. Microemulsions: Overview. J. *Dispersion Sci. Technol*., 18: 301-367.

Schachter, M. 2005. Chemical, P'cokinetic, P'codynamic property of statin. *Fundam. Clin. Pharm*., 19:117-125.

Schwendener, R. and Schott, H. 1996. Lipophilic 1-beta-D-arabinofuranosyl cytosine derivatives in liposomal formations for oral and parenteral antileukemic therapy in the murine L1210 leukemia model. *J. Cancer Res. Clin. Oncol*., 122:723-726.

Shah, M., Chuttani, K., Mishra, A. and Pathak, K. 2011. Oral solid compritol 888 ATO nanosuspension of simvastatin: optimization and bio distribution studies. *Drug Dev. Ind. Pharm*., 37: .526–537

Skehan, P., Storeng, P., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, JT., Bokesch, H., Kenney, S., Boyd, MR. 1990.New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst*., 82:1107–1112

Spernath, A. and Aserin, A. 2006.Microemulsion as carriers for drugs and nutraceuticals. *Adv. Colloid Interface Sci*., 128-130: 47–64.

Srinivas, C. and Sagar, S. 2012. Enhancing the bioavailability of simvastatin using microemulsion drug delivery system. *Asian J. Pharm. Clin. Res*., 5:134-139.

Srinivas, C., Mohd, A., Huidrom, S., Rao, A. and Diwan, P. 2011. Development and validation of simvastatin in microemulsion formulation using RP-HPLC. *Int. J. Pharm. Pharm. Sci*., 4:398-403.

Suresh, G., Manjunath, K., Venkateswarlu, V. and Satyanarayana, V. 2007. Preparation, characterization, and in vitro and in vivo evaluation of lovastatin solid lipid nanoparticles. *AAPS Pharm. Sci. Tech*., 8: E162–E170.

*******