Compendium of Oral Science Volume 9(1)/2022
Original Article

Investigation of the inhibitory effects of simvastatin in RPMI 2650: An *in-vitro* study

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Received: July 09, 2021
Accepted for publication: Aug 27, 2021

DOI: https://doi.org/10.24191/cos.v9i1.16788

ABSTRACT

Objective: *The present study was carried out to investigate the antiproliferative effects of simvastatin involving Human Squamous Nasal Cell Carcinoma (RPMI 2650 cell line), which is one of the most common head and neck cancers with the highest incidence and mortality rates in Asian countries. The anti-cancer effects of simvastatin in NPC have not yet been investigated in depth, hence it will be illustrated in the present study.* **Materials and methods:** *The cells were treated with various concentrations of Simvastatin in a dose dependent (0, 0.1, 0. 4, 3.0 mg/ml) and time dependent (24, 48 and 72 hours) manner. The cancer cell viability was then assessed by using MTT assay at absorbance of 590 nm.* **Results:** *Simvastatin induced reduction in cell viability number and induced apoptosis in RPMI 2650 cell line. Simvastatin for 72 h significantly reduced cell growth as compared to 48 and 24 h pre-incubation with simvastatin treatment. After 72 h incubation with 0.1 mg/ml, 0.4 mg/ml and 3.0 mg/ml simvastatin, the cell viability decreased from 100% in treated control cells to 23%, 21% and 14% respectively.* **Conclusions:** *This finding demonstrate the potential of simvastatin and probably may have therapeutic benefit for NPC cell growth.*

Keywords: *Anticancer, Simvastatin, Human squamous nasal cell carcinoma, Head and neck carcinoma.*

Abbreviations: *ASIR- age-specific incidence rate, ASMR- age-specific mortality rate, DMSO- Dimethyl sulfoxide, EBV- Epstein-Barr virus, EMEM- Eagle's minimum essential medium, FBS- Foetal bovine serum, FPP- farnesylpyrophosphate, GGPP- geranylgeranylpyrophosphate, HMG-CoA - 3-hydroxy-3-methylglutarylcoenzyme CoA, LDL- low-density lipoproteins, MTT- (3-(4,5-dimethylthiazol-2-yl)-2, 5-*

diphenyltetrazolium bromide), NADH- Nicotinamide adenine dinucleotide, NPC- Nasopharyngeal carcinoma, RPMI 2650- Human Squamous Nasal Cell Carcinoma

INTRODUCTION

Nasopharyngeal carcinoma is a non-lymphomatous, squamous-cell carcinoma that arises from epithelial cells lining of the nasopharynx which is also commonly referred to as NPC. This neoplasm shows varying degrees of differentiation and the area of the nasopharynx mostly associated with NPC is the Fossa of Rosenmuller, which lies adjacent to the Eustachian tube openings (Wei *et al.,* 2005). NPC is one of the most common head and neck cancers with the highest incidence and mortality rates in Asian countries. The annual incidence rate is ~20-50 cases per 100,000 individuals. Studies reported the five countries with the highest age-specific incidence rate (ASIR) of nasopharynx cancer were Malaysia, Singapore, Indonesia, Vietnam, and Brunei, and the five countries with the highest age-specific mortality rate (ASMR) were Indonesia, Vietnam, Singapore, Malaysia, and Brunei (Mahdavifar *et al.,* 2016).NPC differs from other head and neck cancers in its aetiology, epidemiology and potential therapeutic options and it is also one of the best examples of a human solid tumor that is consistently associated with a virus and is etiologically related to the Epstein-Barr virus (EBV) (Nakanishi *et al.,* 2017, Pioche-Durieu *et al.,* 2005). Other major etiological factors proposed for NPC pathogenesis include genetic susceptibility and environmental factors. However, challenges still exist in the prevention of disease relapse, treatment of patients with refractory or metastatic NPC. Hence to enhance the long-term overall survival rate of NPC, it is crucial to explore more effective treatment modalities (Ma *et al.,* 2019).

Statins are the cholesterol-lowering drug that reduces cholesterol synthesis by preventing the conversion of 3-hydroxy-3-methylglutarylcoenzyme CoA (HMG-CoA) to mevalonate and are widely used, with a safe and well-tolerated therapeutic agent for treating hypercholesterolemia, atherosclerosis, coronary heart disease and stroke (Hothersall *et al.,* 2006). The useful effect of statins is shown to act on the rate-limiting step in cholesterol biosynthesis which inhibits the mevalonate pathway and reduces the synthesis of geranylgeranyl pyrophosphate and farnesyl pyrophosphate (Shah S.R *et al.,* 2015).

Research suggests that the broad benefits discovered with statins might not be mediated solely by their lipid-lowering properties, however probably through cholesterol-independent or pleiotropic effects like antiproliferative, pro-apoptotic and anti-invasive effects in cancer cells such as in breast, colon, prostate cancer and melanoma (Yamashita M. *et al*., 2010, Sonobe M. *et al.,* 2005).

The useful effects of the HMG CoA reductase inhibitors are usually attributed to their capacity to reduce the endogenous cholesterol synthesis, by competingly inhibiting the principal enzyme involved. Mevalonate is the product of HMG CoA reductase reaction and it is the precursor not only for cholesterol but also for many other nonsteroidal isoprenoid compounds, inhibition of this key enzyme may result in pleiotropic effects (Maciejak, A. et al., 2013).

Simvastatin, a synthetic derivative of lovastatin isolated from the culture filtrate of *Aspergillus terreus* is a prodrug of a specific inhibitor of 3-hydroxy-3-methylglutarylCoA (HMG-CoA) reductase. This compound is a lactone. The dihydroxy acid form converted from simvastatin inhibits markedly HMG-CoA reductase. The chemical structure of simvastatin governs its water solubility, which in turn influences its absorption, distribution, metabolism, and excretion. It is not well absorbed, and less than 5% of an oral dose reaches the systemic circulation. The molecular weight is 418.66 Da with the formula of $C25H38O5$.

Among all statins available, simvastatin has received major attention as the potential of anticarcinogenic due to its lactone profile and previous research that demonstrated significant effects of

simvastatin as an anticancer agent (Mohamed N.A.H *et al.,* 2018). Simvastatin prevented DNA replication and cell proliferation by inducing a G1 block of the cell cycle of the human breast cancer cell line MCF-7 which activated ras oncogene expression. For these reasons, HMG-CoA reductase inhibitors have received increasing attention as pharmacological tools for controlling tumour cell growth (Shen *et al.,* 2015). Although simvastatin has been demonstrated to exert in vitro and in vivo anti-tumour effects on various cancer by inhibiting tumour cell growth and inducing apoptosis, the anti-tumour effects of simvastatin on nasopharyngeal carcinoma have not yet been investigated. In the present study, the effect of simvastatin on the differentiation and proliferation of the RPMI 2650 human tumour osteoblast cell line was examined (Longo et al., 2020).

METHOD

Simvastatin was purchased from Sigma-Aldrich (Merck KGaA, German). RPMI 2650, Eagle's minimum essential medium (EMEM) was purchased from American Type Culture Collection (USA). Foetal bovine serum (FBS), trypsin EDTA and antibiotic antimycotic (×100) were purchased from Gibco, Life Technologies (USA). Dulbecco's phosphate-buffered saline (modified 10×) liquid was purchased from Sigma-Aldrich.

Cell Line Culture and Maintenance

RPMI 2650 squamous cell carcinoma were cultured in EMEM supplemented with 10% FBS and 1% antibiotic anti-mycotic. The cells were maintained in a humidified tissue-culture incubator at 37°C and 5% CO2.The culture medium was changed every three days to maintain healthy growth of cells. Once cells have reached 70 – 80% confluences, the culture medium was discarded from the flask and the cells were washed thrice with phosphate buffered saline (DPBS, USA). Subsequently, trypsin-EDTA 0.25 % was added into the flask until it covered the cell monolayer and was incubated for ten minutes in the CO^2 incubator for cell detachment. This resulted in lifting of the cells off the plastic culture surface. The cells were then detached into 15 mL Falcon tube (USA). The cell suspension was centrifuged at 1500 rpm for 5 min at 27°C to form a cell pellet. After centrifugation, 1 mL of fresh CCM was added into the tube containing cell pellet after the supernatant was discarded. The mixture was re-suspended gently until completely homogenized. The cells were then either re-seeded into culture flasks or transferred to culture plates or wells in preparation for experiments.

Cell Viability Assay

MTT is a colourimetric non-clonogenic assay that measures cell viability in a culture by its metabolic activity. The cell culture is stained with a yellow tetrazolium substrate (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT). Viable cells metabolise the substrate by reduction with NADH (Nicotinamide adenine dinucleotide) producing purple, water insoluble, formazan crystals. Dead cells do not reduce tetrazolium and thus the accumulation of purple colouration is proportional to metabolic activity and surviving cell viability. Formazan crystal, which is the final product of MTT reduction is then being dissolved in DMSO (Dimethyl sulfoxide). Spectrometry reading is automated by the use of plate readers which measure resulting optical density from formazan development by the absorbance of monochromatic light, typically around 570nm. Absorbance values for each plate are indicative of the surviving metabolic activity for that culture (Mohamed NAH, 2020).

RPMI 2650 cells were seeded onto 96 well plate and treated with simvastatin in a dose dependent (0, 0.1, 0.4, 3.0 mg/ml) and time dependent (24, 48 and 72 hours) manner with cells density of $5x10^3$ per well. For *in vitro* administration, simvastatin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and stored at -20˚C. A total of 24 h after seeding, cells were treated with the indicated concentrations of simvastatin for 24, 48 and 72 hours. Cells treated with dimethyl sulfoxide was used as the control.

Upon incubation at specific interval time, 10 µL of MTT assay solution were added to each well and the plates were further incubated for 4 h at 37°C. Then, 100 µL of DMSO was added after 70 µL of media was removed and was incubated again for 30 minutes. Spectrophotometric absorbance at 590 nm was measured with the Tecan Infinite M200 microplate reader (USA).

Statistical analysis

All raw data of main outcomes involving NPC cells proliferation were collected and gathered in Microsoft excel spreadsheet (Version 2013). The data were then exported to Statistical Package for Social Science (SPSS, Version 26.0, IBM) software for data analysis. Descriptive statistics were used to summarise the dataset and continuous variables were reported as mean and standard deviation (SD). A repeated-measures ANOVA was applied to evaluate the time and treatment effects. Post-hoc multiple comparisons were executed when a significant treatment between groups was found. A p-value < 0.05 was considered for significant effects and statistical difference between the treatments.

RESULTS

To determine the inhibitory effect of simvastatin on RPMI 2650, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed. RPMI 2650 cells were treated with various concentrations of simvastatin (0, 0.1, 0.4 and 3 mg/ml) for 24, 48 and 72 h and cell viability were measured by using MTT assay at the absorbance of 590 nm. As illustrated in Figure 1, simvastatin statistically decreased the cell viability in a concentration-dependent manner over the dose range from 0.1 to 3 mg/ml ($P<0.05$). However, there is a slight raised in the number of viable cells of 0.1 and 0.4 mg/ml at 48 h hence it will be discussed in the next section. Overall, Simvastatin for 72 h significantly reduced cell growth as compared to 48 and 24 h pre-incubation with simvastatin treatment. After 72 h incubation with 0.1 mg/ml, 0.4 mg/ml and 3.0 mg/ml simvastatin, the cell viability progressively to 23%, 21% and finally 14% respectively.

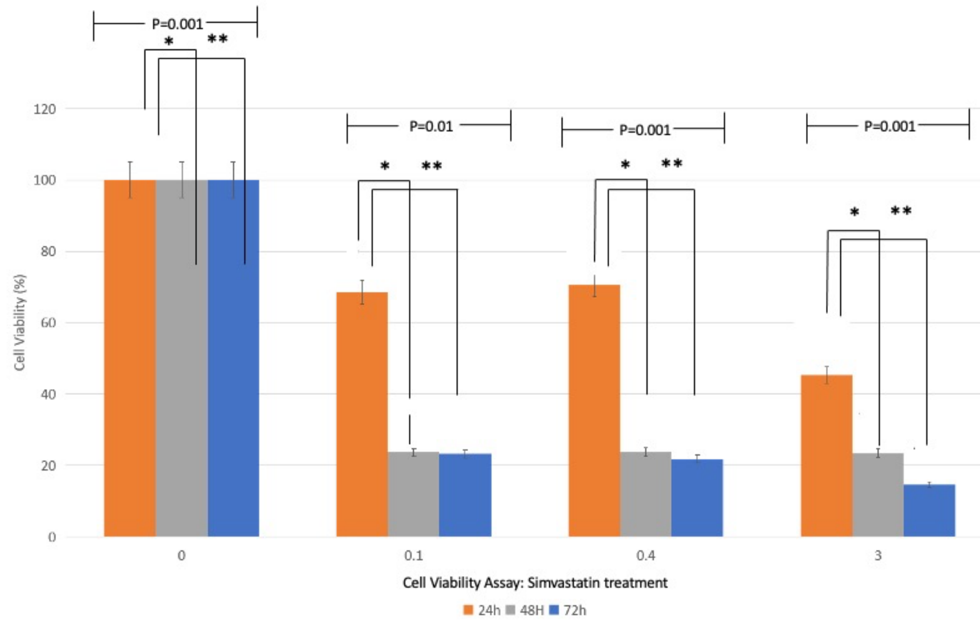

Figure 1: The MTT Assay indicating simvastatin inhibition of RPMI 2650 cells. At higher doses, the proliferative capacity of these cells began to decrease in comparison to control cells. Experiments were performed in triplicate. * indicate the statistic significant level of cells treated with simvastatin within 24 hours, ** 48 hours and ***72 hours

DISCUSSIONS

Nasopharyngeal carcinoma (NPC) may be a rare malignancy in most parts of the world (Chen et al., 2019). Other identified threat factors for NPC are cigarette smoking and occupational exposure to formaldehyde and wood dust (Mimi *et al.,* 2018). Overall prognosis has dramatically improved over the past three decades owing to advances in management and cure rate of NPC has markedly improved with broader application of chemotherapy, and more accurate disease staging of NPC (Tsang RK, 2020).

Several studies were conducted to investigate statin therapy in cancer patients and the hypothesis influence the studies are since statins lower LDL-C, this could influence carcinogenesis and tumour progression. Next, statins reducing prenylation may reduce signaling through pathways involved in carcinogenesis and cancer progression and finally, statins reduce the secretion of pro-inflammatory cytokines, which have been shown to drive carcinogenesis and metastasis (Beckwitt *et al.,* 2018). Simvastatin is the most used lipid-lowering statin drug, which is derived from lovastatin. In previous studies, simvastatin has been shown to exhibit anti-proliferative and apoptotic activity against numerous types of cancer cell lines, including colon, prostate and breast. Thus, this study was conducted with a specific objective which is to investigate the effect of simvastatin as antiproliferative agents on NPC (RPMI 2650 cell line).

In this study, the cells were treated with various concentrations of Simvastatin in a dose-dependent (0, 0.1, 0. 4, 3.0 mg/ml) and time-dependent (24, 48 and 72 hours) manner in triplicates. The results of the present study indicated that simvastatin decreased cell viability in RPMI 2650 cells found to simvastatin

decreased cell viability in C666-1 cells and the statin-induced inhibitory effects were associated with HMG-CoA reductase-dependent apoptosis and cell cycle arrest (Ma *et al.,* 2019). Another study by (Shen *et al.,* 2015) suggested that simvastatin showed marked inhibition of tumor cell proliferation, and there is significant cell cycle arrest at G0/G1 phase (Shen *et al.,* 2015). However, our results showed a slight raised in the number of viable cells between 0.1 and 0.4 mg/ml treatment at 48 h. This might be due to the number of cells within the well that was not homogenised during the cell culture. In this study, the amount of cells used were 10^5 and we hypothesised that the high volume of cells in the well contributed to the non-homogeneity of simvastatin treatment in the RPMI 2650.

CONCLUSION

In conclusion, simvastatin has dose-dependent antiproliferative effect on RPMI 2650 cells *in vitro*. Following to this preliminary studies, it can be postulated that simvastatin may be associated with a reduction in of cell death. Therefore, further work is needed as to investigate the potential of simvastatin as an intervention agent in the treatment of NPC as to elucidate the pathways involved in the necrosis and apoptotic of cell death events treated with simvastatin.

CONFLICT OF INTEREST

The authors would like to declare that there is no conflict of interest.

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