

Full Length Research Paper

Cytotoxic evaluation of fluvastatin and rosuvastatin and effect of fluvastatin in the hela cell cycle

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Accepted 13 May, 2019

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, were originally designed to reduce cholesterol biosynthesis and have been extensively used as prevention drugs against hyperlipidemia and cardiovascular conditions. Recently, these compounds have been shown to display chemopreventive activity against cancer. However, the effects of statins on cancer are not completely understood. For this reason, we have studied the cytotoxic effect of rosuvastatin and fluvastatin on three cell tumoral lines: human larynx carcinoma (HEp-2), human nasopharyngeal carcinoma (KB), and human epithelial carcinoma (HeLa). We have found that only fluvastatin has relevant activity against the tumoral cell lines assayed and the capacity to arrest G₁-phase, whereas a significant decline was observed in the S-phase percentage. Fluvastatin IC₅₀ were 2.43±0.56 µg/mL (HEp-2), 2.29±0.19 µg/mL (KB), and 5.02±1.52 µg/mL (HeLa), whereas rosuvastatin showed poor activity. These results indicate that the cytotoxic effect of fluvastatin may not depend directly on HMG-CoA reductase inhibition. The antitumor statins effect needs further investigation.

Key words: Cell cycle, cytotoxicity, statins.

INTRODUCTION

It has been demonstrated that abnormally high levels of serum cholesterol contribute to atherosclerosis and coronary artery disease. Statins, such as rosuvastatin and fluvastatin are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and efficiently and widely used drugs in the treatment of lipid disorders, especially hypercholesterolemia (Aguilar-Salinas et al., 1993; Watts and Dimmitt, 1999; Mahmoud, 2006; Anand et al., 2009).

In addition to their cholesterol-lowering effects, statins have been reported to display additional pharmacological properties such as antiviral (Bader et al., 2008; Bader and Korba, 2010; Jameson et al., 2010; Milazzo et al., 2010) and antitumor activities (Takahashi et al., 2006;

Takahashi and Nishibori, 2007; Garwood et al., 2010), however the antitumor molecular mechanisms by which the statin block cancer cell growth are poorly understood (Ghosh-Choudhury et al., 2010), and there are emerging interests to explore the anticancer potentials of HMG-CoA reductase inhibitors in the clinical setting, particularly in tumor sites sensitive to these agents *in vitro* (Chan et al., 2003), some mechanism proposes for anticancer activities are apoptotic inducer, antiangiogenic and antimetastatic. These properties are associates with the inhibition of the synthesis of isoprenoid intermediates of the mevalonate pathway (Sławińska and Kandeferszerzeń, 2008). Although several methods have been developed for statin quantification (Campos-Lara and Mendoza-Espinoza, 2008), their pharmacological properties have been disregarded and there is no cytotoxic evaluation in tumoral cell lines for some synthetic statins such as rosuvastatin and fluvastatin, since only exists study for the natural statins such as mevastatina and lovastatin. For this reason, we have studied the cytotoxic effect of rosuvastatin and fluvastatin in three cell tumoral lines: human larynx carcinoma (HEp-2), human

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Abbreviations: HEp-2, human larynx carcinoma; KB, human nasopharyngeal carcinoma; HeLa, human epithelial carcinoma; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

nasopharyngeal carcinoma (KB), and human epithelial carcinoma (HeLa).

MATERIALS AND METHODS

Cytotoxicity assays. HEP-2, KB and HeLa cells were maintained in RPMI 1640 medium with 10% fetal bovine serum and cultured at 37°C in an atmosphere of 5% CO₂ in air (100% humidity). Cells at log phase of their growth cycle were treated in triplicate at various concentrations of the test samples (0.032–20.0 µg/mL), and incubated for 72 h at 37°C in a humidified atmosphere of 5% CO₂. Cell concentrations were determined by the sulforhodamine B method, this colorimetric method expresses the results in grown cell percentage in relation with the control using the following formula, % grown cell = (At-Ab)/(Ac-Ab) x100, where, At= absorbance value of test compound, Ab= absorbance value of blank, Ac=absorbance value of control (Skehan et al., 1990; Philip et al., 1990; Patel and Patel, 2010; Sivacumar and Alagesaboopathi, 2008; Phadungkit et al., 2010).

Statistical analysis

All the assays were set up in triplicate. The results (IC₅₀) were obtained using a nonlinear regression (equation: standard curves, four parameter logistic curve) and expressed as mean ± standard deviation (SD) by the program SigmaPlot (pharmacology, standard curve analysis).

DNA content and cell cycle analysis

DNA content and cell cycle analysis were carried out according to standard procedures (Masuo et al., 1998; Nunez, 2001). HeLa cells in logarithm phase were incubated for 48 h with fluvastatin (IC₅₀ = 10) in DMSO according with the protocol describe for Nunez in 2010. Cells were fixed with cold ethanol 70% (v/v) overnight and stained for 12 h at 4°C with triton X-100 (100 µL), trisodium citrate (100 mg), RNase A (100 mg), propidium iodide (500 µg/mL) and filtered through nylon mesh. This study was carryout in the Centro de Investigaciones y de Estudios Avanzados del Instituto Politécnico Nacional in 2009.

The analysis of the cell cycle was performed in a Becton Dickinson FACSCalibur flow cytometer the number of acquisition was about 20,000 events. Data analysis was performed by using the Watson's model (Watson et al., 1987) implemented in the FlowJo program version 7.2.5.

Statins

Rosuvastatin was obtained from Astra-Zeneca, and fluvastatin was obtained from Sigma-Aldrich in 2008.

RESULTS

Cytotoxicity assays. We examined the cytotoxicity effect of rosuvastatin and fluvastatin on the tumoral lines HEP-2, KB and HeLa. The experiments clearly showed only fluvastatin induced cytotoxicity in the three cell lines in a concentration-dependent manner (Figure 1 and Table 1). Calculated IC₅₀ values are: Fluvastatin IC₅₀ were 2.43±0.56 µg/mL (HEP-2), 2.29±0.19 µg/mL (KB), and

5.02±1.52 µg/mL (HeLa) (Table 1), whereas rosuvastatin have poor activity (IC₅₀ >20 µg/mL).

DNA content and cell cycle analysis

Table 2, illustrates the changes in DNA content distribution in HeLa cell line treated with fluvastatin (Nunez, 2001). The percentage of cells in G₁-phase was higher in the fluvastatin-treated group than in the untreated group, 60.3 % vs 51.3 %, whereas the S-phase was lower in the fluvastatin-treated group than in the untreated one. This statistic analysis was supporter using the Watson model. The differences between the experimental and the control were of 0.2 for fluvastatin and 0.13 for the control.

DISCUSSION

We found that only fluvastatin showed important activity against the three tumoral cell lines assayed (Figure 1). Fluvastatin IC₅₀ were 2.43±0.56 µg/mL (HEP-2), 2.29±0.19 µg/mL (KB), and 5.02±1.52 µg/mL (HeLa). Cell growth was concentration-dependent in the three tumoral cell lines evaluated (Figure 1). Rosuvastatin did not show any significant growth inhibition activity against the tumoral cell lines assayed (Figure 1).

This difference in cytotoxicity may be caused by the chemical differences in the structure. However, Kobayashi et al. (2007) has reported rosuvastatin cytotoxic activity associated with statin increasing intracellular accumulation and related to pH, maybe for the polar character of the molecule this is agree with the poor activity found (Table 1).

Our cytotoxicity results do not support the mechanism suggested by Buchwald and Wachtershauser et al. (1992) who related the statin antitumor activity with HMG-CoA reductase. In spite of being a potent inhibitor of HMG-CoA reductase, rosuvastatin did not inhibit the growth of the cell tumoral lines assayed (Table 1).

Other mechanisms indirectly related with statins have been suggested; i.e. inhibition of *ras* protein activation which is important in the regulation of cell differentiation and proliferation (Goldstein and Brown, 1990). IL-8 induction has been also suggested as its antitumoral action is mediated by activation of natural killer cells and cytotoxic T lymphocytes (Osaki et al, 1999).

These observations suggest that the anticancer effect of statins depends on several factors which may not be directly related with the inhibition of HMG-CoA reductase, as it has been described in previous works (Perchellet et al, 2009). Further experimental research is needed to clarify the statin antitumor mechanism(s).

The flow cytometry study of the effect of fluvastatin on the cell cycle progression in a cervical cancer cell line (HeLa) showed a moderate G₁-phase arrest after 48 h of exposure and a significant decline in the S-phase

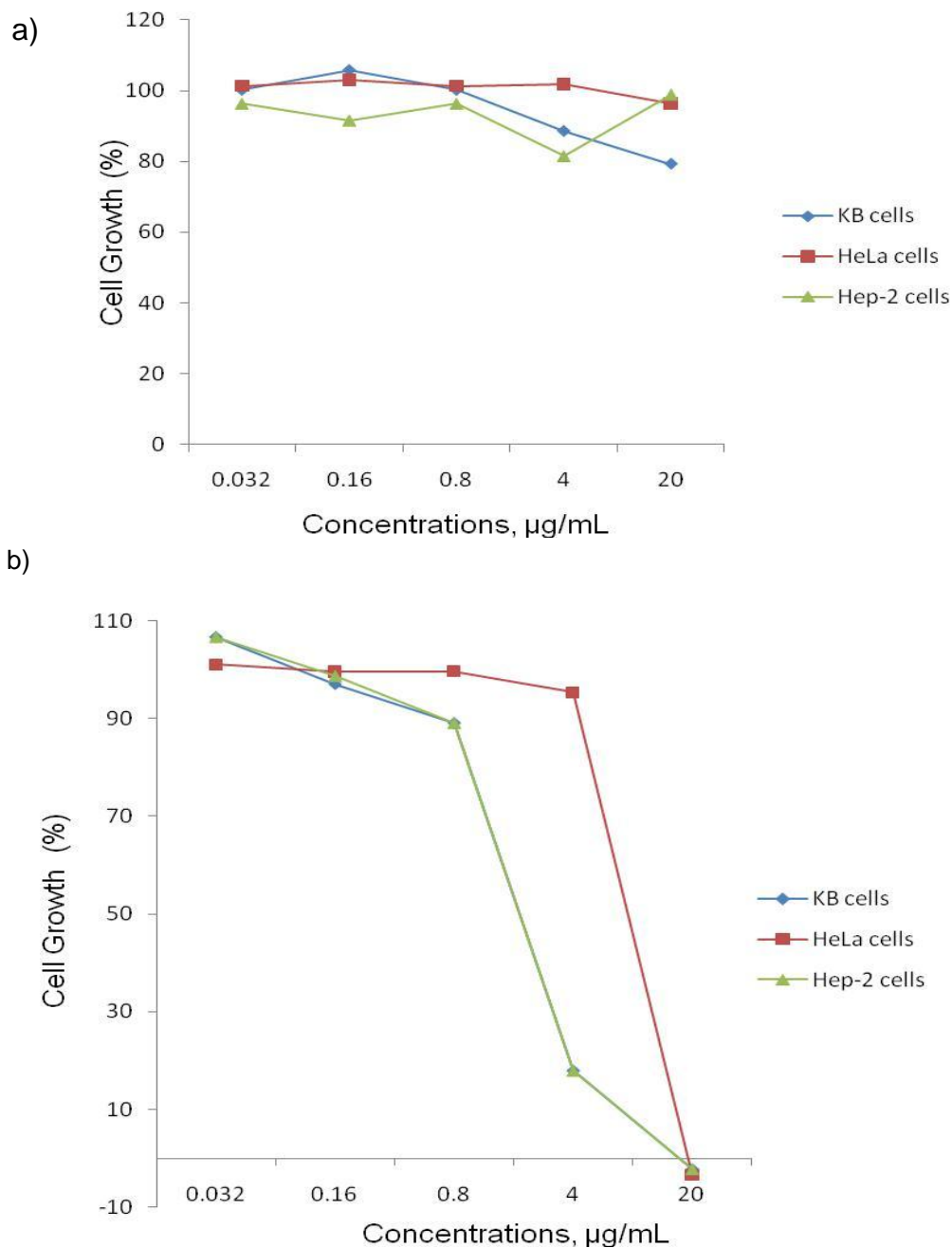


Figure 1. (a) Effect of rosuvastatin on the tumoral cell lines HeLa, KB and HEp-2. (b) Effect of fluvastatin on the tumoral cell lines HeLa, KB and HEp-2. Growth cells percentage was calculate according with the report for Skehan in 1990.

Table 1. Cytotoxic evaluation (IC₅₀, µg/mL).

	HEp-2	KB	HeLa
Fluvastatin	2.43±0.56 ^{1*}	2.29±0.19 ^{1*}	5.02.4±1.52 ^{1*}
Rosuvastatin	>20.00	>20.00	>20.00

¹Standard deviation obtained using the Sigma Plot program sequence (Pharmacology; Standard curve Analysis). *The difference is not statistically significant between the three lines (P=0.01 by unpaired t test).

Table 2. Fluvastatin effects on the cell cycle in the HeLa cell line.

	%G ₁	%S	G ₂	RMS ¹
Fluvastatin	60.3	16.8	10.4	0.20
Control (DMSO)	51.3	29.3	17.1	0.13

¹Differences between experimental data and different model calculations. These calculations were obtained using the Flow job program version 7.2.5.

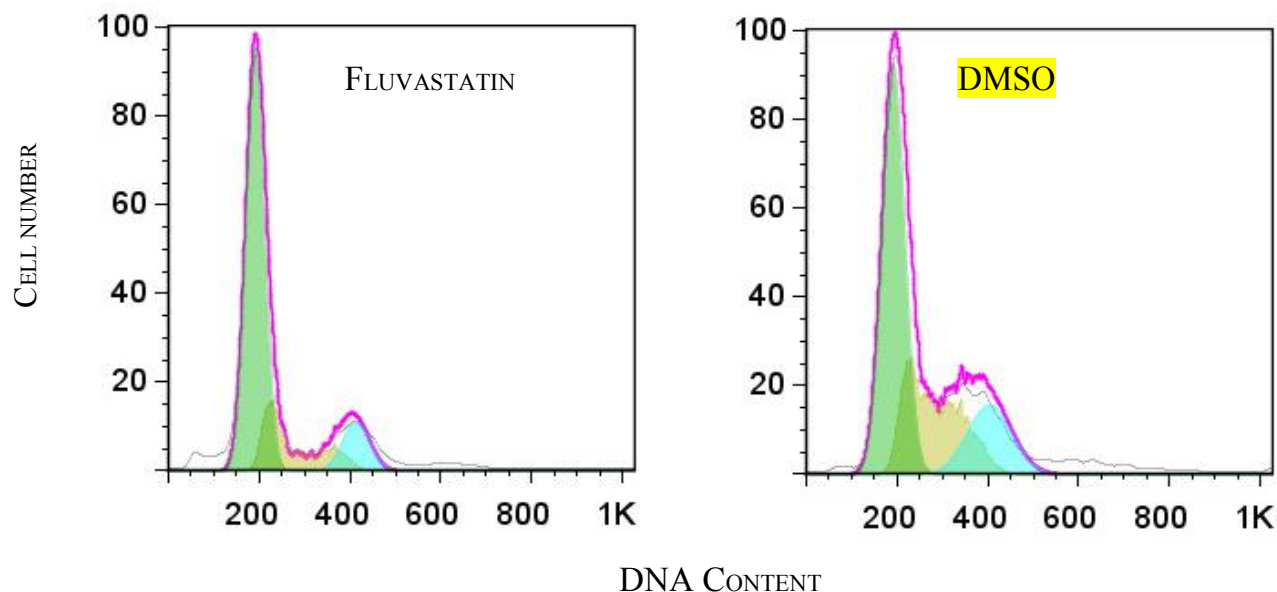


Figure 2. Effect of fluvastatin on the HeLa cell cycle. The results correspond to a representative experiment of three independent assays.

percentage compare with the control (Table 2 and Figure 2). These results are similar to those reported for lovastatin (Cooper, 2002). On the other hand, we have observed a significant increase in the percentage of apoptotic cells, in a similar way to those results reported by Glynn et al. (2008) for lovastatin, mevastatin and simvastatin. In combination with the literature reports (Cooper, 2002; Glynn et al., 2008) this result indicates the importance effect of the statins in the G₁-phase.

In conclusion we have demonstrated the cytotoxic effect of fluvastatin. Our results do not support the hypothesis that the statin antitumor mechanism may be related with the inhibition of HMG-CoA reductase as described in previous works (Buchwald, 1992; Wachtershauser et al., 1992; Sławińska and Kandefer-Szerszeń, 2008). Fluvastatin has been shown to be able to arrest the cellular growth in the G₁-phase as well as to significantly decrease the percentage of cells in the S-phase. Further research is required to examine statin anticancer properties and the relation with the structure chemical.

ACKNOWLEDGMENTS

We highly appreciate the technical assistance of M.C. Victor Rosales García

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