

Neurotoxic effect of statins on mouse neuroblastoma NB2a cell line

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Abstract. – Objective: Evidences from cell culture experiments suggest a link between cholesterol and nervous system disease. Statins may have neurotoxic or neuroprotective effects, but these effects remain controversial. Therefore, the present study was aimed to investigate the possible toxicity of statins on a neurite outgrowth in mouse neuroblastoma NB2a cell line.

Materials and Methods: We have utilized d-cAMP-induced terminally differentiated NB2a cells in culture as an experimental model to study the effects of statins. The cell survival and proliferation were studied by MTT. Measurement of neurite outgrowth was done by neurotoxicity screening test. NB2a cell differentiation was achieved by serum free medium plus 0.5 mM dibutyryl cAMP. Cells were incubated for 24 hours at 37°C. After this period, lovastatin, mevastatin and atorvastatin were added to wells at different concentrations (1, 3, 10, 100 µM). Approximately 100 cells were chosen for each sample and examined randomly 24 hours later, from 10 different fields. Total length of neurite was photographed microscopically and measured by image analyze software. Changes in neurite lengths were expressed as % inhibition compared to that of the control group.

Results: Results showed that three statins at high concentrations induced neurite inhibition, inhibited proliferation and reduced the viability of differentiated neuroblastoma NB2a cells.

Conclusions: Our results suggest that statins could act as a neurotoxic agent at high doses depending upon their concentrations. These results require further investigation at ultra structural and molecular levels to understand long term side effects for clinical safety of statins.

Key Words:

Neuroblastoma NB2a cell line, Neurotoxicity, Statins, MTT.

Introduction

One of the most important risk factors for coronary heart disease is hypercholesterolemia. First choice in the treatment of hypercholesterolemia are the statins. Therefore statins are amongst the most frequently used medications in adults^{1,2}.

Statins exert their anti-hyperlipidemic effects by competitively inhibiting the 3-hydroxy-3-methylglutaryl co-enzyme A (HMGCoA) reductase enzyme. Statins have cholesterol-lowering as well as pleiotropic actions. Pleiotropic effect is defined as the beneficial result of a drug observed besides its expected therapeutic action. These pleiotropic effects have been attributed to the inhibition of HMGCoA reductase enzyme through reduction of mevalonate and other isoprenoid intermediates that form during cholesterol biosynthesis².

Due to their pleiotropic activities, statins can cause cell death by inhibiting the cell cycle and leading the cells to apoptotic pathway^{3,4}. Maltese et al⁴ showed that statins possessed anti-tumor activity in cell cultures. These properties, which appear to be independent of the cholesterol-lowering effects, are similar in both hydrophilic and lipophilic statins⁵.

Even though statins have been hailed as neuroprotective medications largely due to preventing neurotoxicity which develops in response to N-methyl d-aspartate (NMDA) receptors in cultured cortical neurons^{6,7} and decreasing the risk of certain nervous system diseases such as Alzheimer's disease^{8,9}, it has also been shown to inhibit synapse formation, induce synaptic loss, neuritic destabilization and, subsequently, cell death^{10,11}. Though rare, cases have also been reported in which statins exerted neurotoxic consequences such that they might be associated with the development of polyneuropathy^{12,13}.

Neuroprotective^{14,15} and neurotoxic^{16,17} effects of statins are yet to be fully understood. Mevastatin, lovastatin and atorvastatin were chosen for this study to determine whether they cause neurotoxicity, measured by their role in inhibiting neurite outgrowth from differentiating mouse neuroblastoma NB2a cells.

Materials and Methods

Materials

Mouse NB2a neuroblastoma cells were obtained from European Collection of Cell Cultures (ECACC) (cell line: 89121404). Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO, USA). Tissue culture flasks and culture plates were obtained from Falcon/Fred Baker (Runcorn, Cheshire, UK) and gentamicin was purchased from I. Ethem (Genta® 20 mg ampul I. Ethem, Istanbul, Turkey).

Cell Culture

NB2a cells were grown in culture flasks with 5 mL of medium in a humidified 37°C incubator with 5% CO₂. Culture medium for growing NB2a cells consisted of high glucose Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-1, supplemented with 5% (v/v) horse serum, 5% (v/v) foetal bovine serum, 100 units/mL of penicillin plus 100 µg/mL of streptomycin, and 25 µg/mL of gentamicin¹⁸.

Measurement of Neurite Outgrowth, and MTT (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) Metabolism

NB2a cells were plated onto 24 and 96 well culture plates at a cell density of 15,000 cells/mL to measure neurite outgrowth and cellular metabolism, respectively. After 24h, the cells were induced to differentiate and generate neurites in the presence of the statins by the following method: the culture medium was removed from the wells and replaced with serum-free medium plus 0.5 mM dibutyryl cyclic AMP containing mevastatin, lovastatin or atorvastatin at concentrations of 1, 3, 10, and 100 µM for measurement of neurite outgrowth, or 1, 3, 10, 30, 100 µM for measurement of cellular metabolism. Ethanol, which was used as the vehicle served as the positive control. The cells were incubated for a further 24h or 48h, and then the following measurements were carried out¹⁸.

Measurement of Neurite Outgrowth

Cells were fixed in 4% (w/v) formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature, stained for 3 min with Coomassie Blue cell stain (0.6% [w/v] Coomassie Brilliant Blue G in 10% [v/v] acetic acid, 10% [v/v] methanol, and 80% [v/v] PBS), washed with PBS. Samples were photographed and morphometric parameters were measured by three blinded observers using the Olympus BX-40 (Olympus, Tokyo, Japan) light microscope with a video camera (JVC-TK-C 601, Tokyo, Japan) for digital imaging. Images were analyzed by Image-Pro Plus image analyzer (5.1.259, Bioscience Technology, Bethesda, MD, USA). Subsequently, 10 different fields with approximately 10 cells were chosen for each statin drug and control. A software algorithm was written using the functions of the image analyzer available to enable the automatic measurement of the total length of neurites (in pixels) for the cells in a given field and to express the results as the average length of neurites per cell¹⁸.

Measurement of MTT Metabolism

MTT assay, reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to a purple formazan product was used to estimate cell viability and proliferation. Cells were incubated with 0.5 mg/ml of MTT in the last 4h of the culture period tested; the medium was then decanted. Formazan salts were dissolved with dimethylsulphoxide (DMSO) and the absorbance was determined at 570 nm in an UV-visible spectrophotometer multiplate reader (VersaMax®, Molecular Device, El Cajon, CA, USA). Cells were measured three times for the number of viable cells in culture¹⁹.

Statistical Analyses

Data is expressed as mean ± standard error of the mean (SEM) and examined statistically by one-way analysis of variance (ANOVA) with Tukey post hoc testing. A $p < 0.05$ was considered significant.

Results

In the present study, in which we investigated whether lovastatin, mevastatin and atorvastatin had neurotoxic actions, we utilized MTT assay to

show cell viability and neurotoxicity screening test to demonstrate inhibition of neurite outgrowth (Figure 1).

MTT Metabolism

MTT assay is a colorimetric and quantitative method that relies on detecting the color change, which indicates presence of living cells in an environment, by reading the absorbance at a certain wavelength by a spectrophotometer. In the present study, the absorbance of the control group was taken as 100% and measured the relative absorbances of 1, 3, 10, 30 and 100 μM lovastatin, mevastatin and atorvastatin in neuroblastoma NB2a cell culture (Figure 2).

Significant cell loss was not observed at 1 or 3 μM concentrations of atorvastatin and mevastatin ($p > 0.05$). On the other hand, there were significant cell losses, starting at 10 μM concentrations and increasing significantly at 30 and 100 μM concentrations ($p < 0.001$). The toxic effect on cells treated with lovastatin appeared at 3 μM concentration ($p < 0.05$) and increased significantly ($p < 0.001$) and gradually at 10, 30 and 100 μM concentrations (Figure 2).

Neurite Outgrowth

Cells of neuroblastoma cell line differentiate and show neurite outgrowth when serum is re-

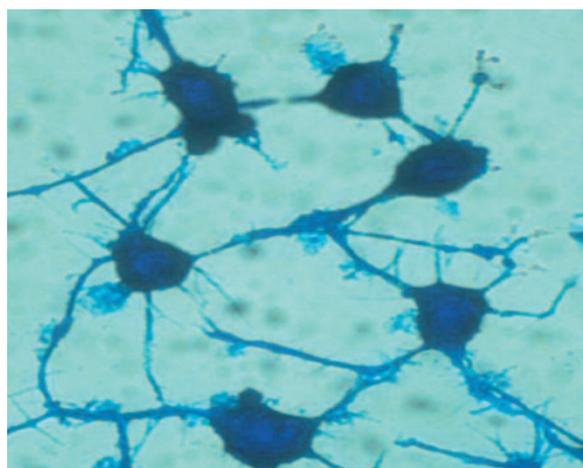


Figure 1. Differentiation NB2a cell to neurons by adding d-cAMP to serum free medium (Coomassie Blue stain, X400).

moved from and d-cAMP is added to the medium (Figure 1). However, if these cells are exposed to toxic effects at this stage, their neurite forming capabilities become disrupted, neurite outgrowth is inhibited or neurites become shortened. We examined the results of 3 separate statine derivatives on neurite outgrowth formation in mouse neuroblastoma NB2a cell line.

The consequences of mevastatin, lovastatin and atorvastatin at concentrations of 1, 3, 10 and

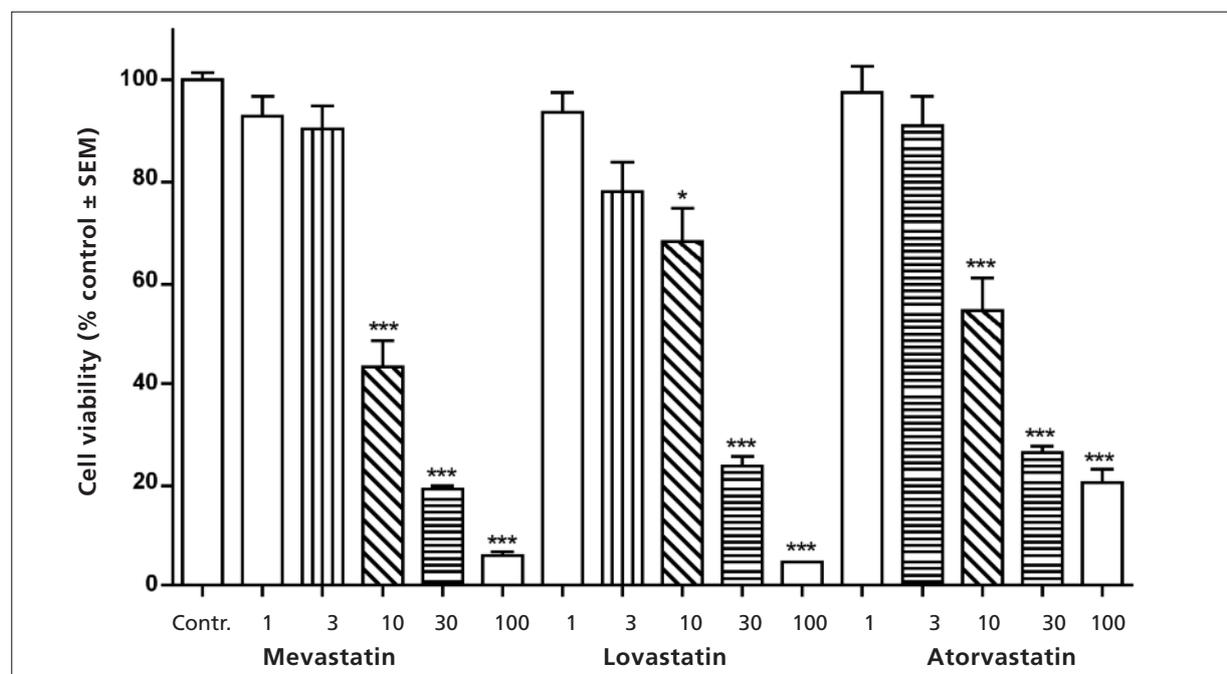


Figure 2. Spectro-photometric MTT measurements with different concentrations of mevastatin, lovastatin and atorvastatin in NB2a cell culture. Results are expressed as mean percentage compared the controls \pm SEM * $p < 0.05$, *** $p < 0.001$.

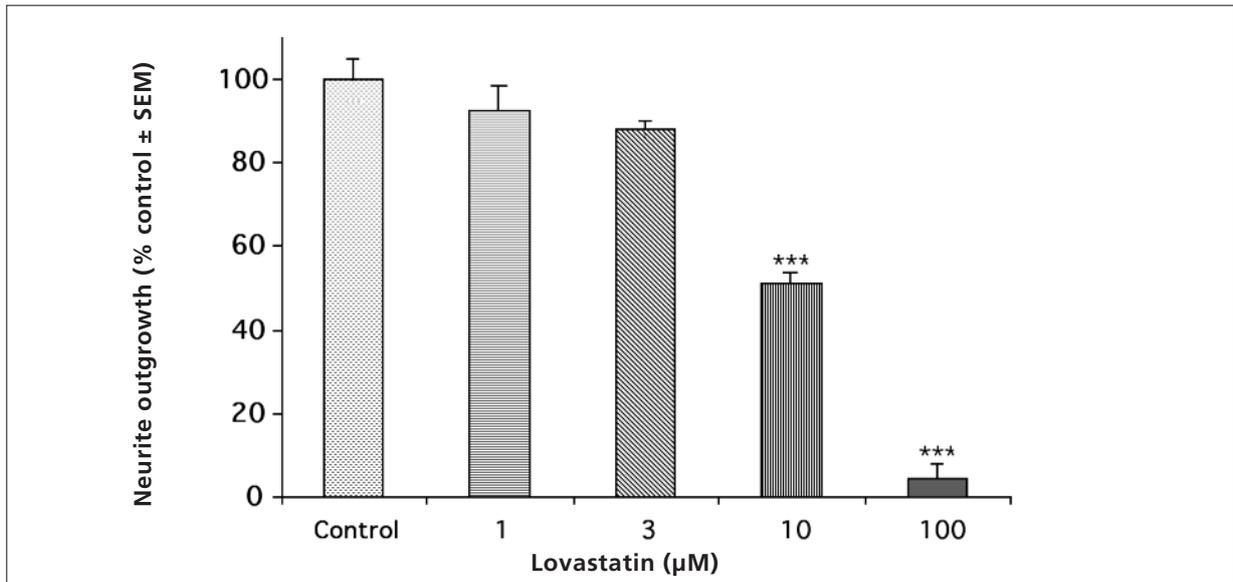


Figure 3. Effects of lovastatin on the neurite outgrowth measured by image analysis. *** $p < 0.001$.

100 µM were investigated on neuroblastoma cell line (NB2a), by forcing differentiation using differentiation medium.

Compared to the control group, there was no significant inhibition in the lovastatin-treated cells at 1 and 3 µM concentrations ($p > 0.05$). At 10 and 100 µM concentrations, on the other hand, there was a marked ($p < 0.001$) neurite inhibition (Figure 3).

Neurite-inhibiting action of mevastatin started at 3 µM concentrations ($p < 0.05$) and this effect was significantly more evident at 10 and 100 µM concentrations ($p < 0.001$). At 1 µM concentration, it did not induce a statistically significant ($p > 0.05$) inhibition as opposed to the control group (Figure 4).

When compared to the control group, we did not observe a statistically significant inhibition in the cells treated with atorvastatin at 1 and 3 µM

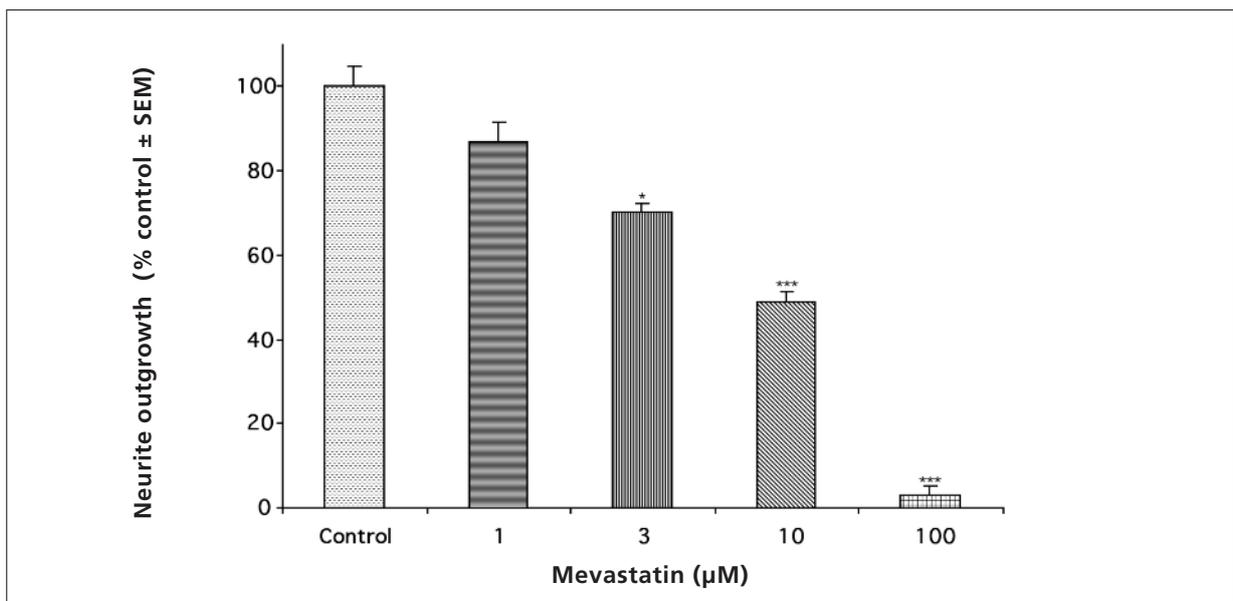


Figure 4. Effects of mevastatin on the neurite outgrowth measured by image analysis. * $p < 0.05$, *** $p < 0.001$

concentrations ($p > 0.05$). At 10 and 100 μM concentrations, they showed, similar to other statins, significant ($p < 0.001$) neurite inhibition at these concentrations (Figure 5).

Discussion

We investigated the role of cholesterol pathway on neurite outgrowth and cell viability, mouse neuroblastoma cells, neurons treated with the HMG-CoA reductase inhibitor, mevastatin, lovastatin and atorvastatin.

Statins are cholesterol-lowering drugs with an open or closed-ring structure. Statins with a closed-ring side chain are converted to open-ring form in the liver by non-enzymatic and enzymatic hydrolysis and inhibit the HMG-CoA reductase activity¹. In cell culture studies, closed-ring statins inhibited cell proliferation while opening statins were ineffective on cell proliferation²⁰. Kumar et al²¹ demonstrated that mevastatin with a closed-ring structure caused degeneration of the differentiated cells and inhibited the growth of neuroblastoma cells in a dose-dependent fashion, whereas pravastatin with an opening structure was not effective on cell growth. Furthermore, they also showed that, by the help of the proliferation medium, almost half of mevastatin was converted from closed-ring to opening structure. In other words, statins with

closed-ring structure can be converted to opening structure as a result of the esterase content of the medium²¹.

Michikawa et al²² reported that in cerebral cortical neuron cultures, mevastatin reduced cholesterol biosynthesis to less than 40% in comparison to the controls at 1 μM concentration, caused death of 50% of the cells at 10 μM concentration and that neuronal cell death was comparable to the control group when mevalonate was given. The results obtained in the present study are in agreement with these studies. This can be explaining by inhibition of the proteasome activity which has been held accountable for the neurotoxic effects of statins^{17,21,23}.

Ludwing et al¹⁷ showed that closed-ring lovastatin inhibited the proteasomal chymotrypsin-like (ChTL) activity in breast cancer cell line and that caused cell death by interrupting the cell-cycle, which was reversed by mevalonate.

The facts that cholesterol plays an essential role in synaptic formation and inhibits dendrite growth selectively can be attributed to the decrease in microtubule stability as a result of inhibition of the MAP-2 phosphorylation. It is known that signaling pathway of Rho GTPase is fundamental in neuronal differentiation²⁴. Actin polymerization and formation of stress fibrils in response to RhoA activation cause retraction of the dendrites and neurites²⁵. The geranylgeranylated protein RhoA appears to be involved in signal-transduction pathways essential for neuronal differentiation²⁶ and

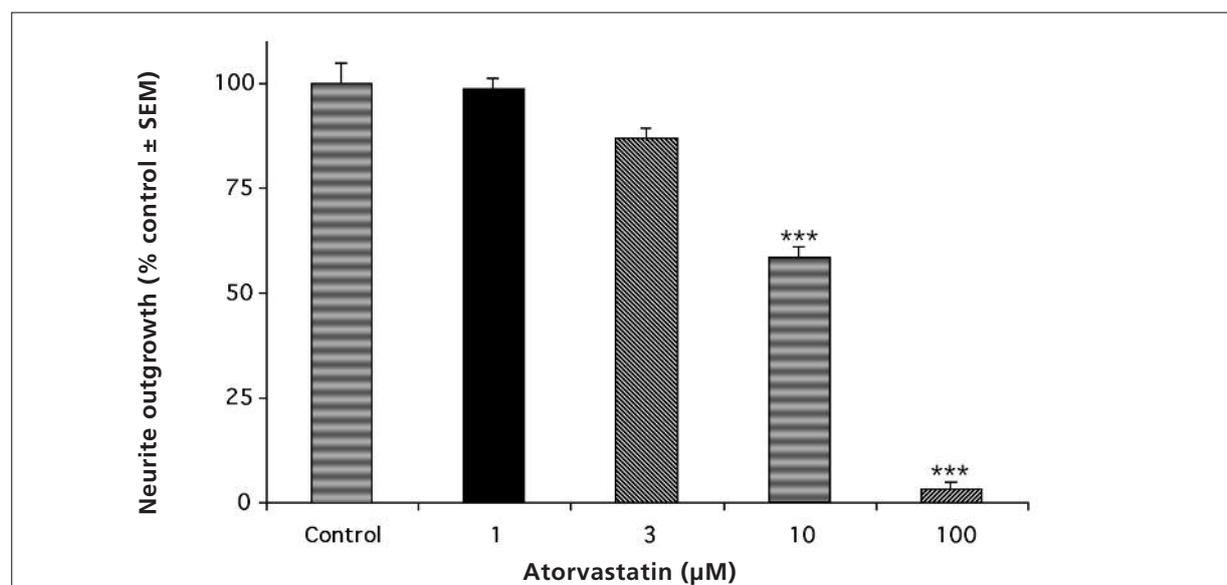


Figure 5. Effects of atorvastatin on the neurite outgrowth measured by image analysis. *** $p < 0.001$.

inhibition of protein prenylation could underlie the action of lovastatin on neurite outgrowth (k5). In the CNS, statins have been found to inhibit synapse formation, to induce synaptic loss, neuritic destabilization, and cell death^{10,11}. Statins might also be associated with the development of polyneuropathy^{12,13}. Moreover, recent clinical reports indicate cognitive deficits associated with statin treatment²⁷. The results of various studies that tried to explain the neurotoxic consequences with different molecular mechanisms might also explain our findings.

Statins have been suggested to cause isoprenoid-dependent differentiation with accompanying neurite outgrowth, but in other studies neurite retraction and cell rounding are observed²⁸. Lovastatin, atorvastatin and simvastatin have been used in SH-SY5Y neuroblastoma cells in which they exerted comparable neuroprotective effects by increasing the nicotinic acetylcholine receptors and decreasing the cholinesterase activity²⁹. Statins have also been shown to inhibit A beta-neurotoxicity^{15,30}. While Kumano et al¹⁶ reported that simvastatin induced neurotoxicity in PC12 neuroblastoma cell line by neurite inhibition and through apoptosis via nerve growth factor (NGF) receptor, tyrosine kinase receptor (Trk), Sato-Suzuki and Muroto showed contradictory evidence that simvastatin acted neurotrophically and induced neurite outgrowth¹⁴. Cholesterol is a key molecule in the CNS²⁵. Neuroprotective or neurotoxic results can be observed, depending on the effects of statins on mevalonate pathway. It is our belief that neurotoxic effects of statins are more dominant, but only at fairly high concentrations.

In conclusion, we noted in our study on mouse neuroblastoma NB2a cell line that lovastatin, mevastatin and atorvastatin caused neurite inhibition and cell death at higher concentrations. The toxic results which appear at higher but not lower concentrations call for caution in terms of drug safety and side effects during prolonged use.

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