

Simvastatin induces proliferation inhibition and apoptosis in C6 glioma cells via c-jun N-terminal kinase

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Abstract

The lipid-lowering drugs, statins, induce apoptosis in a variety of tumor cells. Here we investigated the apoptotic effect of the lipophilic statin, simvastatin, in C6 glioma cells and the underlying effects on intracellular signal transduction. Simvastatin inhibited cell proliferation totally after 20 h of treatment as shown by the decrease in proliferating cell nuclear antigen expression in the nucleus. Subsequently, simvastatin caused apoptotic cell death by shrinkage of cytoplasm and condensation of chromatin, and DNA fragmentation. The features of apoptosis were visible only after 48 h of treatment, possibly reflecting a requirement for cell commitment to growth arrest. In immunocytochemical and immunoblotting experiments we have shown that simvastatin markedly increased the phosphorylation of ATF-2 and c-jun in the nucleus of the C6 glioma cells at early time points which was preserved even 24 h after treatment. In contrast, activities of protein kinases Erk1/2 and AKT in the cell survival pathway remained unchanged throughout the treatment. Selective inhibitor of JNK, but not p38 kinase, reduced simvastatin-induced cell death and ATF-2 and c-jun phosphorylation suggesting that JNK-dependent activation of ATF-2 and c-jun may play an important role in simvastatin-induced proliferation inhibition and apoptosis in C6 glioma cells. These observations suggest that statins may have clinical significance in the prevention of glial tumors beyond their cholesterol-lowering effect and JNK may be a rational target for sensitizing glioma cells to chemotherapeutic agents.

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The statin family of drugs is widely used in the treatment of hypercholesterolemia [11]. They are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the synthesis of cholesterol, which converts HMG-CoA to mevalonate. The statin family is composed of six members: lovastatin, simvastatin, atorvastatin, cerivastatin, pravastatin and fluvastatin. The last two are hydrophilic, whereas the others are lipid soluble, and each member differs in its specificity for HMG-CoA reductase [5].

The clinical use of statins has revealed that these drugs exert beneficial effects on cardiovascular functions that do not

correlate with their ability to lower serum cholesterol levels [12,23]. In addition, antiproliferative and apoptotic effects of statins have been observed in several experimental systems [10,13,20]. These studies implicated that, by limiting mevalonate availability, statins may influence not only the cholesterol levels but also the expression of several proteins, including Ras/Raf/MAP kinases and Rho GTPases. Although inhibition of these G-proteins by impairment of protein isoprenylation has been related to its antiproliferative effect in smooth muscle [17], and cancer cells [9], the molecular mechanisms responsible for the apoptotic and antiproliferative effects of statins are not well understood.

To investigate the signal transduction mechanisms induced by statins in C6 glioma cells we have chosen a lipid soluble statin, simvastatin, since it has been reported that hydrophilic statins were not able to induce cell death, whereas

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all hydrophobic ones were associated with apoptosis in endothelial cells [12]. In the present study, simvastatin time- and concentration dependently reduced cell proliferation and induced apoptotic cell death in C6 glioma cells. These effects induced by simvastatin were shown to be associated with the activation of intracellular signal transduction systems; an increase in phosphorylation of activating transcription factor-2 (ATF-2) and c-jun, suggesting the involvement of c-jun N-terminal kinase (JNK)-dependent cell death pathway.

SB202190, SB203580 and SP600125 were from Calbiochem. Anti-JNK and anti-rabbit alkaline phosphatase conjugated antibodies were from Santa Cruz Biotechnology Inc. Anti-phospho-c-jun, anti-phospho-ATF-2, anti-ATF-2, anti-c-jun, anti-phospho-AKT (Thr-308 and Ser-473) and anti-phospho-ERK antibodies were from New England Biolabs. Anti-PCNA monoclonal antibody was from Zymed Laboratories, USA. Anti-rabbit and anti-mouse biotin conjugated antibodies, streptavidin, biotinylated horseradish peroxidase (HRP) and aminoethylcarbazole (AEC) were from DAKO. Cell culture media, antibiotics and all the other reagents were from Sigma Chemical Co.

C6-glioma cells were grown on tissue culture flasks in Dulbecco's modified Eagle's medium/F12 medium containing 5% heat inactivated fetal bovine serum and antibiotics (100 units/ml penicillin G, 100 µg/ml streptomycin) at 37 °C in 5% CO₂ and 95% air in a humidified incubator.

Cells grown on coverslips were incubated with drugs for different time points as indicated. Coverslips were washed with phosphate-buffered saline (PBS) and fixed with methanol for 5 min at –20 °C. In order to avoid nonspecific immunostaining, cells were incubated with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Following PBS washes, primary antibodies specific to the indicated proteins were applied to coverslips overnight at 4 °C. After washing with PBS, biotin-conjugated secondary antibodies, streptavidin and biotinylated HRP were applied. Sections were developed by using aminoethylcarbazole (AEC) as substrate. Cells were photographed through Olympus BX-50 brightfield microscope and BX-FLA fluorescence attachment using UV filter under 400× and 600× magnification as indicated.

After treatments cells grown in culture dishes were washed once with ice-cold PBS, and lysed in a buffer containing 20 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 10 mM NaF, 0.5 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin. Insoluble material was removed by centrifugation at 13,000 × *g* for 20 min at 4 °C.

An equivalent volume of 2× SDS-sample buffer was added to cell lysates and boiled for 5 min. The supernatants were subjected to electrophoresis on SDS–PAGE gels and transferred to nitrocellulose by using a Bio-Rad apparatus. Membranes were blocked for 1 h at room temperature in PBS containing 3% non-fat dried milk and probed overnight at 4 °C with primary antibodies. The immobilized proteins were detected by using alkaline phosphatase conjugated sec-

ondary antibodies in PBS-3% milk. After washing with PBS, bands were visualized by using BCIP/NBT as substrates.

Cells grown on coverslips were fixed with methanol for 5 min at –20 °C and then washed with PBS. Then cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (0.1 mg/ml) for 15 min at room temperature to allow visualization of the nuclei. Cells were washed and mounted for fluorescence microscopy and photographed through UV filter.

To detect DNA fragmentation genomic DNA was isolated from C6 glioma cells after incubation in the presence or absence of simvastatin at various time points up to 48 h. Briefly, cells were lysed in TE buffer (50 mM Tris–HCl, pH 8.0, and 10 mM EDTA) containing 0.5% sodium lauryl sarcosyl and proteinase K. After chloroform, isopropanol and ethanol precipitation and wash steps running dye was added and preparations were electrophoresed in 1.8% agarose gels in TBE buffer (40 mM Tris–borate and 1 mM EDTA), then ethidium bromide stained DNA fragments were visualized under UV light.

Statins have been shown to inhibit proliferation and to induce apoptosis in a variety of transformed and non-transformed cell types [4,6,10,20]. We have shown here that, exposure of C6 glioma cells to simvastatin resulted in a concentration-dependent (0.5–50 µM) cell death by 50% inhibition at 2.5 µM. This cell death was time-dependent and it was observed only after 48 h of treatment with simvastatin. After 24 h morphological changes in cells treated with simvastatin were observed: they became smaller and rounder. Only after 48 h the cell death induced by simvastatin showed features of apoptosis such as cellular shrinking, chromatin condensation and apoptotic bodies containing nuclear fragments as shown by staining of DNA in the nucleus with DAPI and visualized by fluorescence microscopy (Fig. 1A).

Apoptosis was also verified by detection of DNA fragmentation, which is another classical marker of apoptosis. Consistent with the DAPI staining data in Fig. 1A, simvastatin (10 µM) treated C6 glioma cells showed DNA ladder formation in agarose gel electrophoresis after 48 h of treatment (Fig. 1C).

Analysis of variety of cell types demonstrated that reactive oxygen species may function as intracellular second messengers to induce apoptosis [26]. We have examined whether simvastatin treatment increases free radical generation leading to apoptosis in these cells. The pretreatment of cells with a general antioxidant *N*-acetyl-L-cysteine, which increases level of intracellular glutathione, did not prevent simvastatin-induced cell death in these cells (data not shown) suggesting that oxidative mechanisms may not be involved, albeit this point deserves further investigation.

We investigated whether the apoptotic effect of simvastatin was preceded by the growth arrest of C6 glioma cells. We have detected the expression of the proliferating cell nuclear antigen (PCNA), by immunocytochemical method in C6 glioma cells. PCNA or cyclin, is elevated in the nucleus during late G1 phase and becomes maximal during S-phase

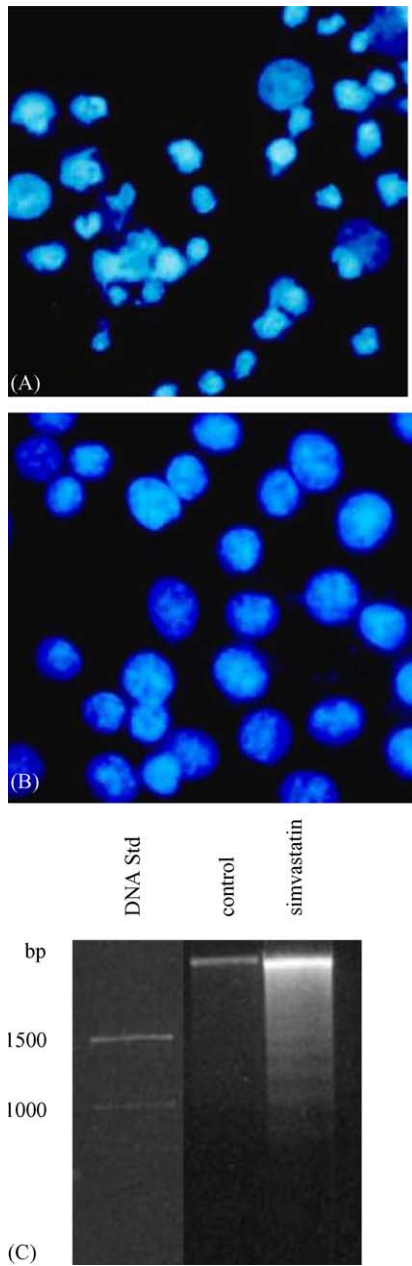


Fig. 1. Simvastatin-induced apoptotic cell death of C6 glioma cells with the shrinkage of cytoplasm and nuclear chromatin condensation. At day 2 after plating cells were incubated with (A) and without (B) simvastatin ($10 \mu\text{M}$) for 48 h. Then they were fixed and stained with DAPI for nuclear visualization and photographed with fluorescence microscope using UV filter under $600\times$ magnification. (C) DNA fragmentation analysis of cell death in C6 glioma cells by agarose gel electrophoresis. Cells treated with simvastatin ($10 \mu\text{M}$) for 48 h, then DNA was extracted and analyzed as described in the text.

and its level correlates directly with rates of cellular proliferation and DNA synthesis. PCNA/cyclin functions as a cofactor of DNA-polymerase to play a fundamental role in the initiation of cell proliferation [18]. Simvastatin caused a concentration-dependent decrease in cell proliferation of C6 glioma cells as detected by immunocytochemical analysis by using antibodies against PCNA protein. At $10 \mu\text{M}$ sim-

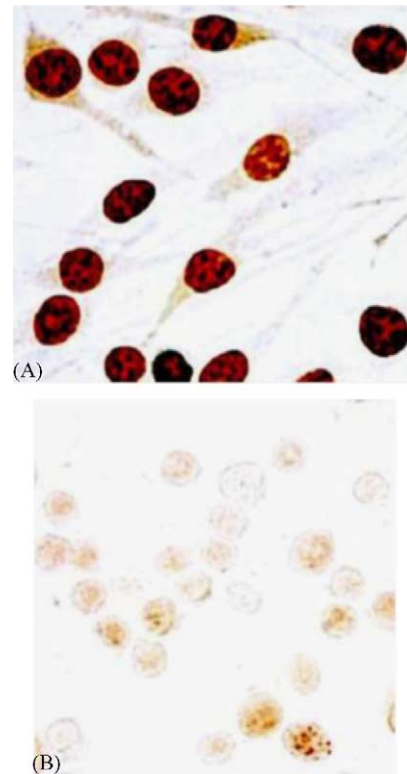


Fig. 2. Inhibition of proliferation of C6 glioma cells by simvastatin as shown by PCNA staining in the nuclei of C6 glioma cells. At day 2 after plating, cells were incubated without (A) and with (B) simvastatin ($10 \mu\text{M}$) for 20 h. Cells were fixed, stained with a monoclonal antibody against PCNA, then visualized by using biotinylated secondary antibody, streptavidin/HRP and AEC chromogen. Cells were photographed through brightfield microscope under $600\times$ magnification.

vastatin concentration, expression of PCNA protein in the nucleus was inhibited nearly totally at 20 h after treatment (Fig. 2B). In parallel, apoptotic death of C6 glial cells was gradually increased up to 48 h. This raises the possibility that, as described in other systems, cells undergoing cell cycle progression are more sensitive to apoptosis by simvastatin.

We have examined the signaling intermediates which may be involved in the apoptosis inducing affect of simvastatin in C6 glioma cells. Hydrophobic statins have been associated with an increase in phosphorylation of JNK, c-jun [14], and Akt [15] in vascular endothelial cells, and with a decrease of phosphatidylinositol-3 kinase (PI3K) activity and phosphorylation of its downstream effector Akt [25] or Erk1/2 [22] in vascular smooth muscle cells with different functional consequences. In immunocytochemical analysis with antibodies against the phosphorylated residues at Ser-63/73 of c-jun and Thr-71 of ATF-2, we found that treatment with simvastatin ($0.5\text{--}50 \mu\text{M}$) increased the phosphorylation of ATF-2 and c-jun in a concentration- and time-dependent manner in the nuclei of the C6 glioma cells. Simvastatin at $10 \mu\text{M}$ concentration increased the phosphorylation of ATF-2 (Fig. 3A) and c-jun (Fig. 3B) within 10 min with sustained phosphorylation up to 24 h after treatment. Western blot analysis by using antibodies recognizing the phosphorylated and native forms of

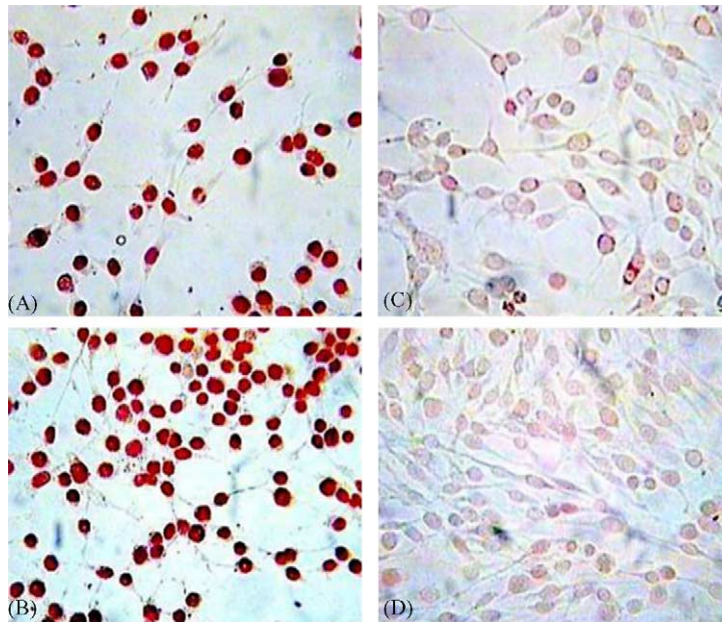


Fig. 3. Stimulation of the phosphorylation of ATF-2 and c-jun by simvastatin (10 μ M) in the nuclei of C6 glioma cells in immunocytochemical analysis. Cells were incubated for 24 h with (A and B) and without (C and D) simvastatin at day 2 after plating. Cells were fixed, stained with polyclonal antibodies against phosphorylated forms of ATF-2 (A and C) and c-jun (B and D). Cells were visualized by using biotinylated secondary antibody, streptavidin/HRP and AEC chromogen. Cells were photographed through brightfield microscope under 400 \times magnification.

ATF-2 and c-jun showed a significant increase in the phosphorylation of these proteins by simvastatin with the similar time course seen in immunocytochemical analysis (Fig. 4 left, upper panels). The increase in the phosphorylation of ATF-2 and c-jun was not due to the increase of the synthesis because in western blot analysis an antibody recognizing ATF-2 and c-jun independently from its phosphorylation status did not detect any increase in the expression of these proteins in simvastatin treated cells (Fig. 4 left, lower panels).

The transactivation capacities of the N-terminal domains of ATF-2 and c-jun can be enhanced through phosphorylation by the MAP kinase members p38 kinase and JNK [3,8]. The involvement of p38 kinase in the induction of apoptosis by simvastatin was excluded by the fact that the specific inhibitors of p38 kinase, SB202190 and SB203580, were not able to reverse the phosphorylation of ATF-2 and also the apoptotic effect of simvastatin, indicating that JNK most likely phosphorylates ATF-2 as well as c-jun. We found that

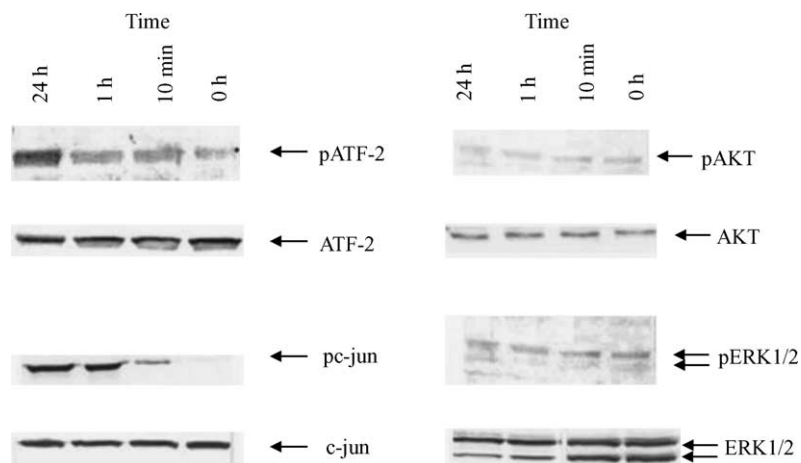


Fig. 4. Stimulation of phosphorylation of ATF-2 and c-jun by simvastatin. Western blot analysis of ATF-2 and c-jun in cell lysates of C6 glioma cells. At day 2 after plating cells were incubated with simvastatin (10 μ M) for the indicated times. Cell lysates were separated by 10% SDS-PAGE, western blotted with polyclonal antibodies against phosphorylated forms of ATF-2 (pATF-2) (left, upper panel), c-jun (pc-jun) (left, lower panel), AKT (pAKT, Thr-308) (right, upper panel) and ERK1/2 (pERK1/2) (right, lower panel), and their corresponding native proteins in separate membranes. Then visualized by using alkaline phosphatase conjugated secondary antibodies and BCIP/NBT as substrates.

inhibiting JNK with a specific inhibitor, SP600125, reduced the levels of ATF-2 and c-jun phosphorylation, but partly protected C6 glioma cells from apoptosis induced by simvastatin. After 24 h, apoptotic morphological changes in cells treated with simvastatin in the presence of SP600125 (20 μ M) were less significant than the cells treated with simvastatin in the absence of SP600125. After 48 h, simvastatin-treated cells in the absence of the JNK inhibitor were totally rounded up and detached from the plate, while in the presence of SP600125, approximately 90% of the cells were viable and processes bearing, but slightly rounded. After 72 h, the apoptotic effect of simvastatin was visible in most of the SP600125 treated cells as stained with DAPI (not shown). These observations indicate that the reversible inhibitor of JNK, SP600125 delays and reduces the toxic effect of simvastatin, however this does not exclude the participation of another pathway in the apoptotic effect of simvastatin. Altogether these results suggest the involvement of JNK pathway in the proapoptotic effect of simvastatin. The expression of JNK was not altered by simvastatin treatment in western blot analysis (not shown).

Since there has been reports indicating the activation of Ras and PI3K pathways by statins [15,17,22], we have examined whether these signaling pathways were also involved in the response of these cells to simvastatin. Immunocytochemical staining and immunoblot analysis using antibodies against the phosphorylated and native forms of these proteins showed that phosphorylation of ERK1/2 and AKT, the downstream effectors of Ras and PI3K, respectively, were unaffected from 10 min to 48 h of simvastatin treatment of cells (Fig. 4 right panels).

Taken together, these data strongly suggest that, simvastatin causes apoptosis and proliferation inhibition by activating, at least partially, the JNK-dependent cell death pathway in C6 glioma cells.

The statin family of drugs inhibits the mevalonate pathway by targeting HMG-CoA reductase and has been shown to be efficacious in treating hypercholesterolemia [11]. Although the well-accepted effects of these drugs are explained by their beneficial actions on the lipid profile, increasing evidence suggests that statins may also exhibit effects unrelated to lipid reduction [23,24]. The potential of this family of drugs has yet to be fully explored.

The present data show that simvastatin induces cell death in C6 glioma cells with characteristics of apoptosis such as, chromatin condensation and laddering, nuclear fragmentation, and shrinkage of cytoplasm. The antiproliferative effect of simvastatin was closely correlated with apoptotic changes in C6 glioma cells. Simvastatin treated C6 glioma cells exhibited typical apoptotic morphology visualized by fluorescent staining of nuclear chromatin with DAPI at 48 h of treatment. This delay may reflect a change in the expression of gene products, such p53, p21/WAF1, Bcl-2, BAD that have been implicated in apoptotic cell death pathways [7].

It has been described in other systems that cells undergoing cell cycle progression are more sensitive to apoptosis. To

test this idea we have investigated the correlation between cell growth and cell death in C6 glioma cells. Proliferation of cells was studied by detecting PCNA expression in the nucleus of glioma cells by immunocytochemical analysis. Simvastatin caused a concentration-dependent decrease in the expression of PCNA in the nucleus of C6 glioma cells indicating the inhibition of proliferation of these cells.

Several studies have shown that lipophilic statins can trigger a variety of cells to undergo apoptosis both in vitro and in vivo [4,9,10,20]. However, the signaling events involved in this effect of statins have not been well defined. We have further investigated the intracellular signaling mechanisms of simvastatin-induced cell death in glioma cells. ATF-2 is a member of the ATF-2/cAMP-response element binding protein family of basic leucine zipper proteins involved in cellular stress response [16]. The transcriptional potential of ATF-2 is induced by NH₂-terminal domain phosphorylation by JNK and p38 kinase which mediates stress responses including DNA damaging events. JNK and p38 kinase-dependent N-terminal phosphorylation of ATF-2 in response to cellular stress or electrical activity has been shown in a variety of cell types [1,2]. In C6 glioma cells, simvastatin strongly increased the phosphorylation of ATF-2 and c-jun via JNK rather than p38 kinase. Simvastatin mediated ATF-2 and c-jun phosphorylation and apoptosis were reduced by treatment with the JNK specific inhibitor SP600125 while the p38 kinase specific inhibitors SB202190 and SB203580 had no effect. These results indicate that JNK-dependent phosphorylation of ATF-2 and c-jun plays an important role in the cell death induced by simvastatin in C6 glioma cells.

The Ras/Raf/MEK/ERK kinase cascades occupy pivotal position in signal transduction processes that send replication signals from the cell's plasma membrane to the nucleus. Also, several studies have suggested that survival signals are mediated by the PI3K-dependent activation of the protein kinase AKT [7]. However, in immunocytochemical and western blot analysis we have found that ERK1/2 and AKT were not activated by simvastatin in C6 glial cells.

JNKs phosphorylate and activate various cellular factors implicated in regulating altered gene expression in cellular survival and proliferation [8,16]. Because these events are commonly associated with the pathogenesis of a number of diseases, the potential of JNK inhibitors as therapeutics has attracted considerable interest [19]. We show in this report that JNK activation by simvastatin in glioma cells induces c-jun and ATF-2 activation. Furthermore, these data suggest that, JNK-mediated signals may be regarded as targets for suppression of proliferation and induction of apoptosis in glial tumors. Components of this cascade provide viable targets for therapeutic intervention in glial tumors, while these non-lipid-related effects of lipophilic statins in glial cells may also be beneficial in treatment of other central nervous system conditions such as, multiple sclerosis and Alzheimer's disease [21].

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