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Atorvastatin augments temozolomide's efficacy in glioblastoma via prenylation-dependent inhibition of Ras signaling

Peng Peng [a, 1], Wei Wei [b, 1], Cheng Long [b, *], Jingwen Li [b, **]

[a] Department of Neurosurgery, Xiangyang Central Hospital, Hubei University of Arts and Science, Xiangyang, 441021, People's Republic of China
[b] Department of Oncology, Xiangyang Central Hospital, Hubei University of Arts and Science, Xiangyang, 441021, People's Republic of China

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ABSTRACT

Ras signaling is often dysregulated and plays essential roles for the maintenance of glioblastoma. The proper function of Ras depends largely on the appropriate post-translational modification termed prenylation. Targeting protein prenylation therefore represents an alternative therapeutic strategy in glioblastoma. In this study, we demonstrate that prenylation inhibition by atorvastatin is active against glioblastoma. Atorvastatin alone dose-dependently inhibits growth and survival of multiple glioblastoma cell lines. Its combination with temozolomide significantly enhances temozolomide's efficacy in *in vitro* cultured cell system as well as *in vivo* xenograft glioblastoma tumor model. We further show that this is achieved by the inhibition of Ras prenylation, leading to decreased activation of Ras and its downstream signaling pathways, including Erk, rS6 and eIF4E. Our findings suggest that inhibition of Ras activity by atorvastatin effectively targets the MEK and other signaling pathways. Our study provides a fundamental evidence to repurpose atorvastatin for a potential treatment of glioblastoma.

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1. Introduction

Glioblastoma is the most common and fatal brain tumor with no effective therapeutic options [1]. It is characterized by the highly mutated genome which results in the deregulation of various oncogenic signaling pathways involving growth, survival and cancer stem cell maintenance [2]. Among these key pathways, Ras is frequently overexpressed and mutated in glioblastoma, and its aberrant activation plays a pivotal role in regulating cell proliferation, differentiation, signal transduction and tumorigenesis [3–5]. Ras proteins at the plasma membrane cycle between an active GTP-bound and an inactive GDP-bound state. However, these small GTPases (eg, Ras superfamily proteins and other regulator proteins) appropriate function largely depend on a post-translational modification prenylation that enables their correct localization into cytoplasmic membrane and interactions with various signal [6].

Statins are HMG-CoA reductase inhibitors and are used to treat patients with hypercholesterolemia [7]. However, statins have been demonstrated to inhibit prenylation in cancer cells and have anti-cancer benefits. Statins inhibit protein prenylation via blocking HMG-CoA reductase that catalyzes the synthesis of mevalonate which serves as the precursor or isoprenoids and cholesterol in the mevalonate pathway [8]. The mevalonate pathway intermediates FPP (farnesyl diphosphate) and GGPP (geranylgeranyl diphosphate) which are essential for prenylation [9]. Besides lowering cholesterol, statins can induce apoptosis and inhibit cell cycle progression in a number of different types of cancers [8,10,11]. Atorvastatin, a member of statins, was shown to suppress invasion and promote apoptosis in glioblastoma spheroids tumor cultured in fibrin gel [12,13].

In this study, we systematically investigated the effect of atorvastatin alone and its combination with temozolomide in glioblastoma using cultured cells and xenograft mouse model, and analysed its molecular mechanism of action. Our results show that atorvastatin is effective in inhibiting growth and survival of glioblastoma via suppressing Ras signaling in a prenylation-dependent manner. In addition, atorvastatin significantly enhances temozolomide's efficacy *in vitro* and *in vivo*.

* Corresponding author.
** Corresponding author. Department of Oncology, Xiangyang Central Hospital, Hubei University of Arts and Science, Jingzhou Street 136, Xiangyang, 441021, People's Republic of China.
E-mail addresses: 645085675@qq.com (C. Long), ljw0607@163.com (J. Li).
[1] These two authors contributed to this work equally.

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2. Materials and methods

2.1. Cells and compounds

Four human glioblastoma cell lines A172, T98G, U87 and U251 were obtained from the American Type Culture Collection (ATCC, US) and maintained according to ATCC instructions. Atorvastatin, geranylgerniol (GGOH), farnesol (FOH), mevalonolactone (MV) and temozolomide were purchased from Sigma and dissolved in DMSO. Compound stocks were prepared at 1000-fold greater than experimental concentrations.

2.2. MTS proliferation assay

Overall cell growth was assessed using the 3-[4,5-dimethylthiazol-2yl]-diphenyltetrazolium bromide (MTT) colorimetric assay (Promega, US). Cells (5000/well) were treated with drug alone or combination on 96-well plate for 3 days prior to proliferation measurement.

2.3. Measurement of viability

Cells (1×10^5/well) were treated with drug alone or combination on 24-well plate for 48 h. Cells were then harvested using trypsin (Sigma, US) and stained with Annexin V-FITC and 7-AAD using Annexin V-FITC/7-AAD Apoptosis Kit (BD Pharmingen, US) according to the manufacturer's instructions. The stained cells were analysed on a Beckman Coulter FC500. Cells with both Annexin V negative and 7-AAD negative staining were considered as viable cells. The percentage of such population was determined by CXP software analysis.

2.4. Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blot (WB) analyses

Cells were treated with drugs for 24 h. Total proteins were lysed by RIPA buffer (Life Technologies Inc, US) supplemented with final concentration of 1X protease inhibitor cocktail solution. Protein concentrations were determined using colorimetric DC Protein assay (Bio Rad, Hercules, CA). Equal amount of proteins were resolved using denaturing SDS–PAGE and analysed by WB using antibodies against p-rS6(S235/236), S6, p-ERK(Thr202/Tyr204), ERK, eIF4E(S209), eIF4E, p-mTOR(2448), mTOR, Rap1A and anti-β-actin (Santa Cruz Biotechnology, US), and Ras (Becton Dickinson). The bands were detected using the chemiluminescence kits (Amersham Biosciences, UK).

2.5. Ras activation ELISA assay

Cells were treated with drugs for 24 h prior to measuring activated Ras levels using Ras activation ELISA Assay Kit (Merck Millipore, US). Briefly, cells were lysed and protein lysis were added to the Raf-1-RBD coated wells. The captured active Ras is detected and measured quantitatively through addition of anti-Ras antibody and HRP conjugated secondary antibody. Positive and negative controls were included.

2.6. Transfection

Plasmid containing human HRAS Q61L was a kind gift from Dr. Dominic Esposito. 2 μg of control and HRAS Q61L plasmids were transfected into U251 cells (1×10^5/well) using Lipofectamine 2000 transfection reagent (Invitrogen) on 12-well plate according to manufacture's instructions. After 48 h transfection, cells were harvested for Ras activation assay or treated with atorvastatin for another 2 or 3 days for proliferation and viability assays.

2.7. In vivo glioblastoma xenograft

In vivo glioblastoma xenograft mouse model was generated by subcutaneous injection of U251 cells. Briefly, 100 μl of PBS containing 10 million U251 cells were subcutaneously injected into the flank of total 24 SCID mice (6-week-old, from Shanghai laboratory animal center, Chinese Academy of Sciences). Mice were randomized into four groups (6 mice/group). When tumor volume reached ~100 mm^3, the mice were treated with vehicle (DMSO/Saline, 20%/80%), intraperitoneal temozolomide at 40 mg/kg three times a week, oral atorvastatin at 50 mg/kg daily, combination of temozolomide and atorvastatin. Tumor volumes were calculated using the formula: length x width^2 x 0.5236. The *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of Hubei College of Arts and Science.

2.8. Statistical analyses

All data are expressed as mean and standard deviation (SD). Statistical analyses were performed by unpaired Student's *t*-test with p-value < 0.05 considered statistically significant.

3. Results

3.1. Atorvastatin inhibits growth and decreases viability in cultured glioblastoma cells and augments temozolomide's efficacy in vitro

To investigate the effects of atorvastatin on cell growth and survival in glioblastoma, we performed MTS proliferation assay and viability assay using flow cytometry for Annexin V/7-AAD on multiple glioblastoma cell lines, including A172, T98G, U87 and U251. These cell lines are derived from different patients with glioblastoma and have been extensively employed as glioblastoma cell models representing various molecular subtypes [14].

We found that atorvastatin inhibited proliferation of A172, T98G, U87 and U251 cells in a dose-dependent manner, with ED_{50} of ~6 μM (Fig. 1A). Atorvastatin also significantly decreased viability of glioblastoma cell lines (Fig. 1B). Importantly, the combination of atorvastatin and temozolomide (a standard drug used in chemotherapy of gliomas) inhibited more proliferation and decreased more viability than atorvastatin or temozolomide alone (Fig. 1C and D), demonstrating that atorvastatin augments temozolomide's inhibitory effects in glioblastoma cells.

3.2. Atorvastatin acts on glioblastoma cell through inhibition of prenylation

We next investigated if atorvastatin's inhibitory effects on the growth and survival of glioblastoma cells were associated with its capacity to inhibit prenylation by analysing prenylation status of small GTP-binding proteins Ras and Rap1A. Inhibition of Ras prenylation can be detected by immunoblotting because its unprenylated forms display reduced mobility in SDS-PAGE compared with their prenylated. In addition, the unprenylated form of Rap1A is recognized by a specific unprenylated anti-Rap1A antibody.

Ras was in the prenylated forms in control cells. The unprenylated form of Ras with reduced electrophoretic mobility was detected at doses as low as 5 μM of atorvastatin in T98G and U251 cells after 24 h drug treatment (Fig. 2A). Consistently, a dose-dependent increase on unprenylated Rap1A level was also detected in T98G and U251 cells exposed to atorvastatin (Fig. 2A), demonstrating that atorvastatin inhibits prenylation in glioblastoma cells.

Geranylgeraniol (GGOH) is metabolized to geranylgeranyl

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