Life Sciences 104 (2014) 38–46

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Oncogenic BRAF inhibitor UAI-201 induces cell cycle arrest and autophagy in BRAF mutant glioma cells

Jun-Ho Ahn [a], Yong Woo Lee [b], Soon Kil Ahn [a,c], Michael Lee [a,*]

[a] Division of Life Sciences, College of Natural Sciences, Incheon National University, Incheon 406-772, Republic of Korea
[b] School of Biomedical Engineering and Sciences, Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA 24061, USA
[c] YOUAI Co., Ltd., Suwon-Si, Gyeonggi-Do 443-766, Republic of Korea

ARTICLE INFO

Article history:
Received 16 January 2014
Accepted 28 March 2014
Available online 8 April 2014

Keywords:
BRAF inhibitor
UAI-201
Glioma
Autophagy
Cell cycle arrest

ABSTRACT

Aims: An activating mutation of BRAF (BRAF-V600E) has been reported in a subset of malignant brain tumors. Thus, the aim of the present study was to identify the antiproliferative effect of the new oncogenic B-Raf targeting drug UAI-201 on 6 types of glioma cell lines with differing B-Raf mutational status.
Main methods: The IC_{50} values of UAI-201 were determined using crystal violet assays in six glioma cell lines. Real-time RT-PCR was performed to assess the functional role of multidrug resistance proteins in response to UAI-201. The effects of UAI-201 on six glioma cells were further examined by immunoblotting analysis, cell cycle analysis, flow cytometric apoptotic assay and autophagy assay. To identify the role of autophagy in UAI-201-induced growth inhibition, Atg5 and Beclin 1 were knocked down by RNA interference.
Key findings: Real-time RT-PCR analysis showed a poor correlation between UAI-201 activity and the expression level of multidrug resistance proteins. The growth inhibitory effects of UAI-201 correlated with the BRAF-V600E genotype of the glioma cell lines. BRAF blockade with UAI-201 resulted in dose-dependent inhibition of MEK/ERK phosphorylations and increased G_0/G_1 arrest in glioma cells with BRAF-V600E. Interestingly, UAI-201 preferentially induced autophagy in BRAF-V600E cells, but not in BRAF-WT cells. More notably, autophagy inhibition through siRNA-mediated Beclin 1 knockdown partially attenuated the growth inhibition induced by UAI-201 in BRAF-V600E cells.
Significance: The pro-death autophagic processes could be one of the underlying mechanisms for the sensitization of BRAF-V600E glioma cells toward UAI-201.

Introduction

Glioma is the most frequent primary central nervous system tumor. Especially, glioblastoma (WHO glioma grade IV) remains one of the most fatal solid cancers comprising 50% of all of the human gliomas. Glioblastoma is not sensitive to the majority of anticancer treatments and is consequently characterized by a dramatically low survival rate among patients (Chang et al., 2006). Although the molecular mechanisms that underlie the development and progression of these tumors are far from being completely understood, some molecular biomarkers, including deletions of chromosome arms 1p and 19q, promoter hypermethylation of the *O*6-methylguanine-methyl-transferase (*MGMT*) gene, and mutation status of the isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* genes, are being used in neuro-oncology for the evaluation of glioma patients (Pollo, 2012).

Activating mutations of *v-raf* murine sarcoma viral oncogene homolog B1 (BRAF) were identified in a large proportion of human cancers (http://www.sanger.ac.uk/genetics/CGP/cosmic). Notably, BRAF somatic missense mutations were observed in 66% of malignant melanomas (Davies et al., 2002), with a valine-to-glutamate substitution (V600E) in the glycine-rich loop being the most frequent BRAF mutation. Dysregulation of the RAF signaling pathway through its constitutive activation has also been shown to be critical to the molecular pathogenesis of gliomas (Lama et al., 2007). BRAF-V600E mutations are characteristic of high-grade gliomas, while KIAA1549-BRAF fusion genes are associated with low-grade astrocytomas. Six percent of glioblastomas, and up to 66% of pleomorphic xanthoastrocytomas, express the BRAF-V600E mutation (Basto et al., 2005; Schindler et al., 2011), while 50–65% of pilocytic astrocytomas express the BRAF fusion mutation BRAF-KIAA1549 (Jones et al., 2008). It has recently been reported that epithelioid glioblastomas showed a high percentage of the BRAF-V600E mutation (Kleinschmidt-DeMasters et al., 2013).

This prevalence of BRAF mutations has led to the development of inhibitors of mutated BRAF (Ribas and Flaherty, 2011). The first BRAF-targeting drug to be accepted for clinical use was sorafenib, which

* Corresponding author at: Division of Life Sciences, College of Life Sciences and Bioengineering, Incheon National University, 12-1 Songdo-dong, Yeonsu-gu, Incheon 406-772, Republic of Korea. Tel.: +82 32 835 8247; fax: +82 32 835 0763.
E-mail address: mikelee@incheon.ac.kr (M. Lee).

http://dx.doi.org/10.1016/j.lfs.2014.03.026
0024-3205/

inhibits both the RAF/MEK/ERK and vascular endothelial growth factor (VEGF) signaling pathways (Wilhelm et al., 2004). However, it was ineffective in tumors in which BRAF was mutated (Eisen et al., 2006). PLX4032 (Vemurafenib) is a recently developed BRAF mutant-specific inhibitor, which inhibits BRAF-V600E with a 10-fold lower half-maximal inhibitory concentration (IC_{50}) than BRAF-WT (Tsai et al., 2008). We recently reported UAI-201 (described as UI-152 in previous reports) as a potent ATP-competitive inhibitor of RAF proteins (Ahn et al., 2012). In biochemical assays, UAI-201 showed a 10-fold higher potency against BRAF-V600E than the well-known oncogenic BRAF inhibitor PLX4032.

To date, studies evaluating the mechanisms and effectiveness of selective BRAF inhibitors in brain tumors have been poorly performed. Here, we provide the first evidence that brain tumors harboring the V600E mutation can be targeted using UAI-201. The present results demonstrate that UAI-201 treatment significantly affects cell viability in BRAF-V600E-positive glioma cell lines, concomitant with the inactivation of the MAPK pathway and the induction of autophagy. Importantly, this is the first study to show the induction of autophagy in glioma cells by UAI-201 treatment. We discuss our findings as a rationale for the potential application of UAI-201 in the treatment of glioma.

Materials and methods

Antibodies and reagents

Rabbit polyclonal anti-Beclin 1, anti-Bcl-2, anti-$p21^{Cip1}$ and anti-$p27^{Kip1}$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For the apoptosis assay, the FITC Annexin V Apoptosis Detection kit was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). SYBR Premix EX TaqII, used for real time PCR, was obtained from Takara Korea Biomedical Inc. (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and penicillin–streptomycin were purchased from GIBCO-Invitrogen (Carlsbad, CA, USA). Verapamil, MK-571 and 3-methyladenine (3-MC) were obtained from Sigma (St. Louis, MO, USA). The BRAF-targeting drug UAI-201 (described as UI-152 in previous reports) was obtained from YOUAI Co. Ltd. (Suwon-Si, Gyeonggi-Do, Korea). The structural formula was previously reported (Ahn et al., 2012).

Cell lines and cell culture

The glioma cell lines (A172, T98G, U-87-MG, KG-1-C, NMC-G1 and DBTRG-05MG) were obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea), the Japan Health Sciences Foundation Health Science Research Resources Bank (Osaka, Japan) or the American Type Culture Collection (Manassas, VA, USA). All the cell lines were maintained at 37 °C in DMEM supplemented with 10% FCS, penicillin–streptomycin, and glutamine. For experimental purposes, cells were cultured in 60-mm tissue culture dishes until they reached ~80% confluency.

siRNA transfection and chemical treatment

For Beclin 1 knockdown, a pool of 3 target-specific siRNAs and a non-targeting siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The targeted sequences were: CAGCUCAACGUCACUGAAAtt, GAGAUCUUAGAGCAAAUGAtt and GGAUGACAGUGAACAGUUAtt. The siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) in Opti-Minimal Essential Medium I (Opti-MEM I) medium (Invitrogen) according to the manufacturer's protocol. After 24 h, the transfected cells were treated with chemical. UAI-201 was dissolved in DMSO and freshly diluted for each experiment. DMSO concentrations were less than 0.1% in all experiments.

Crystal violet assay

The cells were plated in triplicate into 24-well plates at 1×10^4 cells/well and cultured. On day 3, the medium in each well was replaced with the medium containing UAI-201. Each concentration of UAI-201 was applied in triplicate to three separate wells containing cells. On day 7, the cells were fixed with 3.7% formaldehyde for 30 min, washed with water, and stained with 1% crystal violet in water for 30 min. After thoroughly rinsing with water, the plates were dried and crystal violet from the stained cells in each well was extracted with 0.5 ml of a solution containing 0.02 M HCl in 50% ethanol. The absorbance of the samples against a background control (extraction solution alone), as a blank, was measured at 570 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Real-time quantitative reverse transcription PCR analysis

The mRNA levels of multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein (MRP) were measured by real-time RT-PCR. Using reverse transcriptase, cDNA was synthesized from 1 μg of total RNA. Aliquots of cDNA were used as a template for real-time PCR reactions containing either primers for MDR1 and MRP (MRP1, MRP3 and MRP5) or primers for β-actin. The primers were synthesized by Bioneer (Daejeon, Korea) with the following oligonucleotide sequence: *mdr-1*, 5′-CAGGAACCTGTATTGTTTGCCACCAC-3′ and 5′-TGCTTCTGCC CACCACTCAACTG-3; *mrp-1*, 5′-ACCCTAATCCCTGCCCAGAG-3′ and 5′-CGCATTCCTTCTTCCAGTTC-3; *mrp-3*, 5′-CGCCTGTTTTTCTGGTGGTT-3′ and 5′-TTGTGTCGTGCCGTCTGCTT-3; *mrp-5*, 5′-CCAAGCTGACCCCCAA AATGAAAAA-3′ and 5′-TGGATGTGCTTGCCTTCTTCCTCTTC-3′. Real-time PCR was carried out with an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix EX TaqII. The real-time PCR data were normalized for differences in β-actin levels by analysis with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Quantitation of autophagy

The cells were grown on chamber slides, washed with PBS, and fixed in 10% formalin solution for 10 min. Cells were transfected with pEGFP-LC3 (Addgene, Cambridge, MA, USA) for 48 h and then treated with UAI-201 for 24 h. Fixed cells were classified as cells with predominantly diffuse GFP-LC3 fluorescence or a punctate GFP-LC3 pattern using a Zeiss Axio Scope.A1 epifluorescence microscope. To quantify the percentage of cells undergoing autophagy, 200 cells from each group were counted in two independent fields, and the percentages of GFP-LC3-positive cells with GFP-LC3 dots were determined.

Cell cycle assay

The cells were washed with PBS once, trypsinized, and collected by centrifugation at 400 ×*g* for 5 min. The cells (10^6 cells per sample) were fixed with 70% ethanol, and stained with 50 μg/ml propidium iodide (Becton Dickinson) for 5 min. Cell cycle distribution was examined by measuring DNA content using a Gallios flow cytometer and Kaluza analysis software (Beckman Coulter, Inc., Brea, CA, USA). A minimum of 10^4 cells per data point was examined.

Annexin V-propidium iodide double staining

Phosphatidylserine translocation to the outer leaflet of the plasma membrane was assessed by reaction with Annexin V-FITC and detected by a flow cytometer. After treatment, 2×10^6 cells were harvested, washed with ice-cold PBS, resuspended in 200 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and incubated with 5 μl of Annexin V conjugated with FITC for 10 min at room temperature in the dark. Samples were washed with binding buffer,