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The inhibitory effect of CIL-102 on the growth of human astrocytoma cells is mediated by the generation of reactive oxygen species and induction of ERK1/2 MAPK

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ABSTRACT

CIL-102 (1-[4-(furo[2,3-b]quinolin-4-ylamino)phenyl]ethanone) is the major active agent of the alkaloid derivative of *Camptotheca acuminata*, with multiple pharmacological activities, including anticancer effects and promotion of apoptosis. The mechanism by which CIL-102 inhibits growth remains poorly understood in human astrocytoma cells. Herein, we investigated the molecular mechanisms by which CIL-102 affects the generation of reactive oxygen species (ROS) and cell cycle G2/M arrest in glioma cells. Treatment of U87 cells with 1.0 μM CIL-102 resulted in phosphorylation of extracellular signal-related kinase (ERK1/2), downregulation of cell cycle-related proteins (cyclin A, cyclin B, cyclin D1, and cdk1), and phosphorylation of cdk1Tyr^{15} and Cdc25cSer^{216}. Furthermore, treatment with the ERK1/2 inhibitor PD98059 abolished CIL-102-induced Cdc25cSer^{216} expression and reversed CIL-102-inhibited cdk1 activation. In addition, *N*-acetyl cysteine (NAC), an ROS scavenger, blocked cell cycle G2/M arrest and phosphorylation of ERK1/2 and Cdc25cSer^{216} in U87 cells. CIL-102-mediated ERK1/2 and ROS production, and cell cycle arrest were blocked by treatment with specific inhibitors. In conclusion, we have identified a novel CIL-102-inhibited proliferation in U87 cells by activating the ERK1/2 and Cdc25cSer^{216} cell cycle-related proteins and inducing ROS production; this might be a new mechanism in human astrocytoma cells.

Introduction

Camptothecin (CPT) is an alkaloid originally isolated from the bark and stem of *Camptotheca acuminata* (Camptotheca, "Happy Tree"), a tree native to China. Extensive documentation indicates that CPT inhibits the DNA enzyme topoisomerase I, induces apoptosis, interacts with DNA to form a complex, and inhibits DNA, RNA, and protein synthesis (Chen et al., 2002; Chen et al., 2003; Tseng et al., 2008). Because of these effects, synthetic and medicinal chemists have developed numerous syntheses of CPT and various derivatives to improve the benefits of its use in cancer chemotherapy (Chen et al., 2002; Zhao et al., 2005). A number of furo[2,3-b]quinoline derivatives, such as CIL-102, have also been synthesized and have been found to exhibit antitumor effects (Chen et al., 2005; Zhao et al., 2005). In particular, recent studies have indicated that CIL-102 may possess antitumor activity against cancers of the prostate, colon, and breast, as well as cervical carcinoma. This is evidenced by the inhibition of tubulin polymerization followed by apoptosis executed via the caspase and non-caspase pathways (Huang et al., 2005). However, the mechanism by which CIL-102 initiates cell cycle arrest and how the signal cascades become activated remain poorly understood.

The mitogen-activated protein kinases (MAPKs) are a family of protein kinases that transfer signals of stimuli from the cell membrane to the nucleus (Thomas and Huganir, 2004). Intracellular signaling pathways for apoptosis have mainly focused on 2 cascades, the kinase cascade and the protease cascade (Raman et al., 2007). The kinase signaling cascade was originally identified as an important pathway in the transduction of apoptotic signals initiated by stress or anticancer drug stimuli (Morrison et al., 1994). Disruption of signal transduction in a glioblastoma occurs through overexpression of receptor tyrosine kinases, such as epidermal growth factor receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor (Cuevas et al., 2007). These abnormalities lead to constitutive activation of Ras/extracellular signal-regulated kinase (ERK) and PI3K/Akt/mammalian target of rapamycin (mTOR) and have been associated with glioma malignancy (Sunayama et al., 2010).

Reactive oxygen species (ROS) are thought to participate in a wide variety of cellular functions, including cell proliferation,

Abbreviations: ERK, extracellular signal-related kinase; Cdks, cyclin-dependent kinases; ROS, reactive oxygen species.

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differentiation, and apoptosis (Valko et al., 2007). On the other hand, several observations have suggested that ROS and mitochondria might mediate apoptosis induction under both physiologic and pathologic conditions. The elevation of ROS, in excess of the buffering capacity designed to modulate ROS levels, results in potentially cytotoxic oxidative stress. Mitochondria are both sources and targets of ROS (Newmeyer and Ferguson-Miller, 2003). Phenolic phytochemicals inhibit the proliferation of cancer cells, and some are able to generate ROS (Kyriakis and Avruch, 2001). However, the effect of CIL-102-mediated ROS production on human gliomas remains unclear.

Chemopreventive compounds may inhibit cell proliferation through modulation of the MAPK pathways (Valko et al., 2007). Major participants in this kinase cascade are 2 members of the Ras/ERK and PI3K/Akt/mTOR pathways (Le et al., 2003; Min, 2010). Recent studies have demonstrated the essential role of the kinase cascade, as well as its downstream mitochondrial-dependent ROS production pathway, during apoptosis induction by cellular stress and chemopreventive compounds. In the present study, we investigated the molecular mechanisms underlying CIL-102-induced ROS production and activation of kinase cascades in human gliomas.

Glioblastoma is a grade IV astrocytoma, as defined by the World Health Organization; it is a common malignant disease worldwide and is characterized by malignant central nervous system tumors (Louis et al., 2007). The poor prognosis for human malignant gliomas is due to their invasiveness and high rate of recurrence. However, chemotherapy treatments for gliomas are often ineffective because of the intrinsic chemoresistance of these tumors. Therefore, it is imperative to develop more effective drugs. In the present study, we investigated whether CIL-102 inhibits glioma proliferation by activating the ERK1/2 and Cdc25cSer^{216} pathways and whether this activation is related to ROS production. We also attempted to elucidate the signaling steps leading from CIL-102 stimulation of the glioma to cell cycle arrest underlying CIL-102-induced expression of cyclin B, cdk1, and cdk1Tyr^{15}, as well as to G2/M arrest within the glioma cells.

Materials and methods

Chemical reagents and antibodies

All culture materials were purchased from Gibco (Grand Island, NY, USA). 1-[4-(Furo[2,3-b]quinolin-4-ylamino)phenyl]ethanone (CIL-102), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ROS scavenger (*N*-acetyl cysteine [NAC]), 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA), dihydroethidium (DHE), ERK inhibitor (PD98059), Janus kinase (JNK) inhibitor (SP600125), p38 inhibitor (SB203580), and mTOR inhibitor (rapamycin) were purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal antibodies against cyclin A, cyclin D1, cyclin E, cyclin B, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against cdk2, ERK1/2Thr^{202}Ty^{r204}, Cdc25cSer^{216}, and cdk1Tyr^{15} and mouse monoclonal cdk1 antibody were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

The C6 and U87 glioma cells were purchased from the American Tissue Culture Collection (ATCC, USA). C6 and U87 glioma cells were grown in minimum essential medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, and 1% antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin). We purchased passage number 1 of human normal astrocytes (HNA) from ScienCell Research Laboratories (Carlsbad, CA) and cells were grown in astrocyte medium.

Cell growth and proliferation assay

The previously reported MTT quantitative colorimetric assay was verified as being capable of detecting viable cells and was used for cell viability determinations. The cells were seeded at a density of 2×10^4 cells/mL and incubated with CIL-102 at various concentrations for 24 h and 48 h. Thereafter, the medium was changed and incubated with MTT (0.5 mg/mL) for 4 h. The viable cell number is directly proportional to the production of formazan, which can be measured spectrophotometrically at 563 nm, following solubilization with isopropanol. Cell growth was determined by counting the cells at the indicated time points with a Coulter counter, combined with a trypan blue (0.2%) exclusion assay (Kuo et al., 2006a).

Apoptosis assay and cell cycle distribution analysis

Changes in cell morphological characteristics during apoptosis were examined using fluorescence microscopy of 4′,6-diamidino-2-phenylindole (DAPI)-stained cells. The monolayer of cells was fixed with 4% paraformaldehyde for 30 min at room temperature. The fixed cells were permeabilized with 3 treatments in 0.2% Triton X-100 in phosphate-buffered saline, followed by incubation with 1 μg/mL of DAPI for 30 min. The apoptotic nuclei were detected under 200× magnification using a fluorescent microscope with a 340/380 nm excitation filter and were scored according to the percentage of apoptotic nuclei found in samples containing 200 to 300 cells. Flow cytometric analysis of the CIL-102-treated cells was performed using FACScan™ (Becton Dickinson Immunocytometry Systems, UK) (Kuo et al., 2006b). Cell-cycle distribution was analyzed by flow cytometry. Cells stained with propidium iodide were analyzed with a FACScalibur™ (Becton Dickinson), and the data were analyzed by using a mod-fit cell cycle analysis program.

Preparation of total cell extracts and immunoblot analyses

Cells were lysed with a buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor mixture (phenylmethylsulfonyl fluoride, aprotinin, and sodium orthovanadate). The total cell lysate (50 μg of protein) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12% running, 4% stacking) and analyzed by using the designated antibodies and the Western-Light chemiluminescent detection system (Bio-Rad, Hercules, CA), as previously described (Chiu et al., 2011).

Measurement of ROS

The intracellular accumulation of ROS was determined by using the fluorescent probe H_2DCFDA, which is commonly used to measure H_2O_2, but is now accepted as also being sensitive to other peroxides. The oxidation-sensitive dyes DHE (2 μM) or H_2DCFDA (2 μM) was added separately to all tubes 15 min prior to harvest. Incubation was terminated by 10-fold dilution with ice-cold fluorescence-activated cell sorting (FACS) buffer and the cells were washed prior to FACS analysis. ROS (O_2^-) generation was determined through increases in DHE. The fluorescence was measured at the desired time intervals using flow cytometry (Hsu et al., 2007).

DNA plasmid and siRNA transfection

The dominant negative mutants of Ras (RasN17) have been previously described (Chiang et al., 2011). The pSV-β-galactosidase plasmid was co-transfected to normalize transfection efficiency. Before treatment with CIL-102, cells were transfected with an empty vector or with the dominant negative mutant of Ras (RasN17).