Aurora kinase A inhibitor TCS7010 demonstrates pro-apoptotic effect through the unfolded protein response pathway in HCT116 colon cancer cells

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Abstract. Aurora kinase A (AURKA) is essential for regulating mitosis and is frequently amplified in various cancer cell types. However, the effect of AURKA inhibition on the induction of apoptosis remains unclear. In the present study, it was reported that treatment with TCS7010, a specific inhibitor of AURKA, resulted in the accumulation of cells in the sub-G₀/G₁ phase of the cell cycle and increased the percentage of annexin V-binding cells. The cleavage of caspase-2, caspase-7, and poly(ADP-ribose)polymerase (PARP) significantly increased in a time-dependent manner following TCS7010 treatment. In addition, TCS7010 resulted in the production of reactive oxygen species (ROS) and stimulation of the unfolded protein response (UPR), leading to the upregulation of CCAAT/enhancer-binding protein-homologous protein (CHOP), and its downstream target BCL2 like 11 (BIM). Pretreatment with N-acetylcystein, a ROS scavenger, significantly abrogated TCS7010-induced accumulation of CHOP, BIM, cleaved caspase-7 and cleaved PARP. These results suggest that TCS7010 triggers apoptosis through the ROS-mediated UPR signaling pathway.

Introduction

Aurora kinases (AURK) are members of the mitotic serine/threonine kinase family, which includes AURK A, B, and C. Among these kinases, AURKA is frequently amplified in various cancer cell types and can act as an oncogene (1). AURKA performs an essential role in centrosome maturation, chromosome alignment and proper formation of the mitotic spindle (2,3). Inhibition of AURKA has been demonstrated to arrest the cell cycle at the G₂/M phase and suppress tumor growth in vivo (4–7). In prostate cancer cells, knockdown of AURKA expression by shRNA induces apoptosis through an upregulation of tumor protein p53 and its downstream target apoptosis regulator Bax (Bax) (8). However, the specific mechanism underlying the induction of apoptosis via AURKA inhibition remains unclear.

The endoplasmic reticulum (ER) is an organelle that has numerous cellular functions, including protein folding, post-translational modifications and trafficking. Cellular conditions associated with the accumulation of misfolded or unfolded proteins in the lumen of the ER induce a series of ER stress responses, known as the unfolded protein response (UPR) (9). Thus, this allows for restoration of protein folding homeostasis by inhibiting further protein translation, degrading misfolded proteins and increasing the synthesis of molecular chaperones to augment ER folding capacity (9). The UPR signaling pathway is mediated by ER transmembrane proteins, including double-stranded RNA-activated protein kinase-like ER kinase, inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (9). However, if the ER stress is too severe and prolonged for the cell to overcome, the UPR signaling pathway can activate the caspase cascade and thus promote the cell death program through apoptosis (10).

The aim of the present study was to investigate the effects of TCS7010, a specific inhibitor of AURKA, on the induction of UPR-mediated apoptosis. The results demonstrated that TCS7010 produces reactive oxygen species (ROS) and triggers apoptosis through ROS-dependent UPR-mediated pathway.

Materials and methods

Cell culture and chemicals. The human colon carcinoma HCT116 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Cellgro; Corning Incorporated, Corning, NY, USA) at 37°C in a humidified incubator with an atmosphere containing 5%
CO₂. The cells were seeded onto 60-mm culture dishes at a density of 4x10⁴ cells/dish and were further cultured until the cells reached up to a subconfluent monolayer (70-80% confluency). The cells were treated with the stated concentrations of TCS7010 (Tocris Bioscience, Bristol, UK).

Immunoblot analysis. Cells were harvested and lysed for 30 min at 4°C in a buffer consisting of 20 mM HEPES (pH 7.2), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were measured with a Pierce™ Bicinchoninic Protein Assay Kit (#23225; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein extracts (20 µg each) were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Following protein transfer, membranes were washed in Tris Buffered Saline with Tween 20 (TBST; Cell Signaling Technology, Inc., Danvers, MA, USA) 3 times for 5 min each at room temperature, followed by blocking in 5% non-fat milk for 30 min at room temperature. The blots were incubated with primary antibodies for 2 h at room temperature. Antibodies against phospho-Aurora A(Thr288)/B(Thr232)/C(Thr198) (#2914; 1:1,000 dilution), Cleaved caspase-7 (#9491; 1:1,000 dilution), IRE1 (#2394; 1:2,000 dilution), CHOP (#2895; 1:1,000 dilution), Bim (#2819; 1:1,000 dilution), Caspase-2 (#611022; 1:1,000 dilution) were purchased from Cell Signaling Technology, Inc. Antibodies against GAPDH (#sc-32233; 1:5,000 dilution), PARP (#sc-7150; 1:4,000 dilution) were obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA) and antibody against Caspase-2 (#611022; 1:1,000 dilution) was from BD Biosciences (San Jose, CA, USA). Following washing 3 times for 5 min each with TBST at room temperature, horseradish peroxidase-conjugated secondary antibody was incubated for 1 h at room temperature. Secondary antibody to anti-mouse IgG (Goat; #BR170-6516; 1:2,000 dilution) and anti-rabbit IgG (Goat; #BR170-6515; 1:2,000 dilution) were purchased from Bio-Rad Laboratories, Inc., (Hercules, CA, USA). Following incubation with secondary antibodies, membranes were washed 5 times with TBST for 5 min each at room temperature and the immunoreactivity of protein bands was visualized and incubated with a NucleoCounter NC-3000 instrument as described previously (12). Data are representative of ≥3 separate experiments.

Detection of intracellular ROS levels. Cells were trypsinized and incubated with 10 µM H2-2', 7'-dichlorofluorescin diacetate (DCF-DA) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 60 min, followed by incubation with 5 µM TCS7010 for 3, 6, or 12 h. The fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD Biosciences), as described previously (11). CellQuest Pro software version 5.2 (BD Biosciences) was used for acquisition and analysis of the data. Data are representative of ≥3 separate experiments.

Analysis of X-box binding protein 1 (XBP-1) splicing. Total RNA was isolated using the phenol/guanidine-based Isol-RNA Lysis reagent (5 PRIME GmbH, Hamburg, Germany), and the synthesis of cDNA was performed using an iScript cDNA Synthesis kit (Bio-Rad, Inc.). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using XBP-1-specific primers (forward, 5'-AGAGTACGACTCACAGTC3' and reverse, 5'-CATTAATGGCTTCAGCT TG-3') that correspond to nt 277-817, encompassing the IRE-1 cleavage site, as previously described (12). PCR products were further digested for 2 h at 37°C using PstI, whose recognition site is located at nt 556 within the 26-nit intron (nt 531-556) that is lost following IRE-1-mediated splicing (13). The PCR product of unspliced XBP-1 (uXBP1) mRNA is expected to be cleaved into two fragments of 279 bp and 261 bp following PstI digestion, while that of spliced XBP-1 (sXBP1) mRNA is resistant to PstI digestion, producing a 540-bp band (11). Data are representative of ≥3 separate experiments.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by Sidak’s multiple comparisons test using GraphPad Prism software (version 7.0; GraphPad Software Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results and discussion

Effect of TCS7010 on the phosphorylation of AURKA in HCT116 cells. AURKA is overexpressed in a broad range
Immunoblot analysis was performed to evaluate whether TCS7010 inhibits AURKA in HCT116 colon cancer cells. The phosphorylation of all AURK subtypes (A, B and C) was highly elevated at the basal status (Fig. 1B). It was demonstrated that TCS7010 selectively inhibited AURKA phosphorylation on threonine-288 within 15 min of treatment (Fig. 1B).

Effect of TCS7010 on the cell viability and cell cycle progression of HCT116 cells. To assess the cytotoxic effect of TCS7010, exponentially growing HCT116 cells were exposed to different TCS7010 concentrations for 24 h, and cell viability was measured using CCK-8. Treatment with TCS7010 significantly reduced HCT116 cell viability in a dose-dependent manner (Fig. 1C). The effective dose causing 50% of maximal cytotoxicity (ED$_{50}$) was ~5 µM. To investigate the mechanism underlying TCS7010-induced cytotoxicity, the effect of TCS7010 on cell cycle progression was evaluated. HCT116 cells were treated with 5 µM TCS7010 for 24 h and cell cycle progression was monitored using flow cytometry. The number of G1 phase cells decreased (50.7 to 24.3%), with a concomitant increase in the number of sub-G1 phase cells (2.10 to 42.0%), which is indicative of a hypodiploid DNA cell population undergoing apoptosis (Fig. 1D). These results suggest that TCS7010 has a cytotoxic effect against HCT116 colon cancer cells, possibly via the induction of apoptosis.

Effect of TCS7010 on the induction of apoptosis. To determine whether TCS7010 triggers apoptosis, annexin V staining was performed. In the early stages of apoptosis, phosphatidylserine (PS) in the inner side of the plasma membrane translocates to the outer surface. As annexin V preferentially binds to PS (15), detection of PS using annexin V is widely used to identify apoptotic cells in response to cytotoxic agents. PI, which cannot enter live cells, was used to counterstain dead cells. HCT116 cells were treated with 5 µM TCS7010 for 24 h and then incubated with FITC-conjugated annexin V and PI. In Fig. 2A, the lower left quadrant of each cytogram (PI and FITC double-negative) represents viable cells, the lower right quadrant (PI negative/FITC positive) represents early apoptotic cells, and the upper right quadrant (PI and FITC double-positive) represents late apoptotic and dead cells. The PI-negative/FITC-positive population was increased in the cells treated with TCS7010 compared with the untreated control group (16 vs. 48%) following, suggesting that TCS7010 triggers apoptosis in HCT116 cells.

Caspases are a family of cysteine proteases that have an essential role in the progression of apoptosis (16). The effect of TCS7010 on the induction of caspase activation was determined in the present study. Upon TCS7010 treatment, the pro-caspase-2 level gradually decreased in a time-dependent manner and the amount of cleaved active caspase-7, and cleaved poly (ADP-ribose) polymerase (PARP), a well-known marker of apoptosis, increased (Fig. 2B).
substrate for caspase-7, increased in a time-dependent manner (Fig. 2B). These data suggest that caspase activation is involved in TCS7010-induced apoptosis.

**Effect of TCS7010 on the production of ROS.** ROS are chemically reactive molecules containing oxygen, including superoxide anion radical, hydrogen peroxide and the highly reactive hydroxyl radical, all of which are formed as a byproduct of normal cellular metabolism. Abnormally excessive ROS levels may result in cellular damage, known as oxidative stress. Thus, under normal physiological conditions, various antioxidant enzymes, including catalase and superoxide dismutase maintain the proper cellular redox states. However, there is growing evidence for the beneficial effects of ROS on chemotherapy (17,18). As numerous cancer cells are more sensitive to ROS compared with normal cells, ROS production could be used for the selective targeting of cancer cells (12,19-21).

As ROS activates the caspase signaling pathway (22), ROS production levels were analyzed using a fluorescent DCF-DA probe. Upon TCS7010 treatment, ROS accumulation was detectable at 3 h and maintained up to 12 h following TCS7010 treatment (Fig. 2C). These data suggested that ROS may act as an upstream signal in TCS7010-induced apoptosis.

**Effect of TCS7010 on the activation of the UPR signaling pathway.** ROS can induce ER stress (23), and ER stress can trigger apoptosis through the UPR signaling pathway (24,25). Thus, whether TCS7010 activates the UPR signaling pathway was determined in the present study. A typical feature of the ER stress response is the accumulation of UPR sensor proteins. It was demonstrated that the amount of IRE1α protein significantly increased within 3 h compared with no treatment and then returned to the basal level at 24 h following TCS7010 treatment (3 h, P=0.0002; Sidak's multiple comparisons test; n=3; Fig. 3A and B). Activated IRE1 functions as a site-specific endoribonuclease that catalyzes the excision of an unconventional 26-nt intron from uXBP1 to generate sXBP1 mRNA (26). To investigate whether TCS7010 induces XBP1 mRNA splicing, RT-PCR was performed using primers that...
amplify the region spanning the 26-nt intron, followed by digestion with PstI. As the PstI restriction site is within the intron site, the uXBPI PCR product should be cleaved into two fragments (279 and 261 bp) by PstI digestion, whereas the sXBPI PCR product lacking the PstI site should produce a 540-bp band (Fig. 3C). As illustrated in Fig. 3D, TCS7010 caused the accumulation of a shifted PCR band (540 bp) between 1 and 6 h, reflecting the splicing of XBPI mRNA. These data suggest that TCS7010 activates the ER stress-induced UPR signaling pathway.

**Effect of TCS7010 on the accumulation of CHOP and BIM proteins.** Accumulating evidence has demonstrated that CHOP performs an essential role in ER-stress-induced apoptosis via upregulation of the pro-apoptotic protein BIM (24,25,27,28). BIM is a Bcl-2 homology domain 3 (BH3)-containing pro-apoptotic Bcl-2 family member. In general, BIM cooperates with other BH3-only proteins, including truncated BH3 interacting domain death agonist and BAX, to trigger mitochondria-mediated apoptosis (29).

To determine whether CHOP expression is upregulated by TCS7010, HCT116 cells were treated with TCS7010 for different periods of time. Immunoblot analysis demonstrated that CHOP protein expression was maximal at 6 h following TCS7010 treatment and were maintained up to 24 h (Fig. 4A). The amount of BIM protein, a CHOP target, peaked at 12 h and then decreased (Fig. 4A). Densitometric analysis revealed that the increases of CHOP and BIM levels were significant (all P<0.0001; Sidak's multiple comparisons test; n=3) upon TCS7010 treatment. These results suggest that the UPR signaling pathway may be involved in TCS7010-induced apoptosis.
Effect of N-acetylcystein (NAC) on TCS7010-induced CHOP accumulation. The association between ROS accumulation by TCS7010 and the UPR response was determined. Fig. 4B demonstrates that pretreatment of HCT116 cells with 2 mM NAC, a thiol-containing ROS scavenger, significantly abrogated TCS7010-induced CHOP accumulation compared with cells treated with TCS7010 alone (P=0.0055; Sidak's multiple comparisons test; n=3). Furthermore, pretreatment with NAC significantly reduced the TCS7010-induced accumulation of BIM and cleaved caspase-7 compared with cells treated with TCS7010 alone (P=0.0004 and P<0.0001, respectively; Sidak's multiple comparisons test; n=3; Fig. 4C). In addition, the TCS7010-induced cleavage of PARP was significantly reduced by pretreatment with NAC compared with cells treated with TCS7010 alone (P=0.0003; Sidak's multiple comparisons test; n=3). These results suggest that ROS production by TCS7010 is upstream of UPR-mediated apoptosis.

In conclusion, the results of the present study demonstrated that TCS7010 induces ROS-mediated apoptosis. Upregulation of BIM through the UPR signaling pathway may have an important role in TCS7010-induced apoptosis. Currently, the mechanism underlying AURKA regulation of the UPR signaling pathway remains unclear. Additional studies are required to investigate the role of AURKA in the control of ER stress responses.
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