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5′-nitro-indirubinoxime induces G2/M cell cycle arrest and apoptosis in human KB oral carcinoma cells

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ABSTRACT

Our previous study demonstrated that the novel indirubin derivative, 5′-nitro-indirubinoxime (5′-NIO), effectively arrested the tumor growth through the inhibition of cell proliferation and the induction of apoptosis. However, the precise molecular mechanisms underlying 5′-NIO-induced antitumor activity remain unclear. Here, we report that 5′-NIO inhibits the proliferation of human KB oral carcinoma cells via the cell cycle arrest in G2/M phase. 5′-NIO reduced the activity of Cdc2/cyclin B complex through the inhibition of the PLK1 expression. Partially, 5′-NIO also arrested cell cycle in G1/S phase via the reduction of CDK4 and cyclin D1/D3 levels by p16 and induction of the level of $p21^{waf1}$. Using flow cytometry analysis, we showed that 5′-NIO-induced cell cycle arrest is followed by apoptosis. We determined further that 5′-NIO-induced apoptosis is accomplished by the mitochondria-dependent activation of the caspase cascade. Overall, these observations suggest the potential value of 5′-NIO as a candidate for a therapeutic modality for the treatment of oral cancer.

1. Introduction

Recently, the use of plant materials as antitumor agents has gained a great deal of attention for its possible therapeutic qualities. Among the variety of naturally occurring plants, Danggui Longhui Wan attracted the attention of cancer investigators due to its tumor-preventing properties against human malignancies [1–4]. In particular, the therapeutic effects of indirubin isolated from Danggui Longhui Wan were reported in neurodegenerative disorders, inflammation, and chronic myelogenous leukemia [5–8].

Several studies have recently demonstrated that indirubin derivatives have antitumor activity on a variety of human cancer cells through the inhibition of cyclin-dependent kinases (CDKs) or Stat3 signaling [9–13]. The most common indirubin derivative, indirubin-3′-monoxime, has evidenced a marked growth inhibition of cancer cells in a cell culture system. Indirubin-3′-monoxime was documented to inhibit several kinases, including CDK, glycogen synthase kinase-3β (GSK3β), and c-Jun NH_2-terminal protein kinase (JNK), which possess anti-inflammatory effects and block the abnormal tau phosphorylation signaling pathway [5,14–16].

Recently, we synthesized the novel indirubin derivative, 5′-nitro-indirubinoxime (5′-NIO), and demonstrated that it has more potent antitumor activity than does indirubin-3′-monoxime, both *in vitro* and *in vivo* [17]. 5′-NIO effectively arrested tumor growth via the inhibition of cell proliferation and the induction of apoptosis [17]. However, the precise molecular mechanisms by which 5′-NIO induces its antitumor effects remain to be clearly understood.

In this study, we have assessed the molecular action mechanism of 5′-NIO in human KB oral carcinoma cells,

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and demonstrated that 5′-NIO induces cell cycle arrest both in the G1/S and G2/M phases via the downregulation of several key cell cycle regulatory proteins, including CDK4, cyclin D1, cyclin D3, and polo-like kinase 1 (PLK1) via the upregulation of the cyclin-dependent kinase inhibitors (CDKIs) p16 and $p21^{waf1}$. We also demonstrated that 5′-NIO induces apoptotic cell death by promoting caspase activity via a mitochondria-mediated pathway. These results provide insight into the molecular mechanisms of 5′-NIO, in addition to its potential value as a novel candidate as an antitumor agent.

2. Materials and methods

2.1. Cell culture

Human KB oral carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained at 37 °C with 5% CO_2 in Minimum Essential Medium supplemented with 1× MEM non-essential amino acids solution, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum.

2.2. Cell proliferation and cytotoxicity assay

Cell proliferation was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye uptake method, as previously described [18]. In brief, cells were cultured overnight and treated with indirubin or indirubin derivatives (dissolved in 0.1% DMSO). Twenty-four hours later, Cells were washed twice with ice-cold PBS, and 0.25 ml of cell culture medium and 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/ml in PBS) were added. After 3 h of incubation, the medium was removed and 125 μl of acid-isopropanol (0.04 mol/L HCl in isopropanol) were added. The absorbance was measured at a wavelength of 570 nm.

Cell toxicity was evaluated via the measurement of lactate dehydrogenase (LDH) activity after treatment with 5′-NIO using a CytoTox 96® non-radioactive assay kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Optical density was assessed at a wavelength of 490 nm.

2.3. Cell cycle analysis

Cells were harvested and fixed in 100% ethanol for 1 h at −20 °C. After washing in cold PBS, cells were incubated with DNase-free RNase and propidium iodide for 30 min at 37 °C. Samples were then analyzed by flow cytometry using Cell Lab Quanta™ SC (Beckman Coulter, Fullerton, CA).

2.4. Western blot analysis

KB cells were treated with 5′-NIO for 24 h. Cells were washed twice with ice-cold PBS and lysed in RIPA buffer. The protein contents of the cell extracts were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). The proteins (20 μg) were then resolved by SDS–polyacrylamide gel, electrotransferred onto PVDF membrane and blotted with antibodies against cyclin D1, cyclin D3, CDK4, CDK6, p15, p16, p21, caspase-7, caspase-3, phospho-Cdc2 (Cell Signaling Technology, Danver, MA, USA), p53, cytochrome C (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or actin (Abcam, Cambridge, UK).

2.5. PLK1 and Cdc2/cyclin B complex assay

The kinase activity of PLK1 and the Cdc2/cyclin B complex was measured using a CycLex® Polo-like kinase 1 assay kit and a CycLex® Cdc2/cyclin B kinase assay kit (CycLex Co. Ltd., Nagano, Japan) according to the manufacturer's instructions. The absorbance, which represents the amount of phosphorylated substrate, was measured at a wavelength of 450 nm.

2.6. siRNA experiment

The PLK1 siRNA construct was obtained as Silencer® select validated siRNA (Applied Biosystems, Foster City, CA, USA). Cells were transfected with 50 nM of siRNA in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) using X-tremeGENE siRNA Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instruction. Cells were harvested 24 h after transfection. The cell lysates were separated by SDS–PAGE and the expression level of PLK1 was analyzed by Western blot analysis as described above.

2.7. Cell death evaluation

KB cells were treated with 5′-NIO for 24 h. Cells were labeled with Annexin V-FITC and propidium iodide using the Annexin-V-FLUOS staining kit (Roche Molecular Biochemicals) according to the manufacturer's instruction. Apoptotic cells were visualized with a Nikon (Tokyo, Japan) Eclipse E800 automated fluorescent microscope. For flow cytometric analysis, cells were measured with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using Cell Quest software. Apoptosis was also measured using a cell death detection ELISA kit (Roche Molecular Biochemicals). Relative apoptosis that correlates with absorption at 405 nm with a reference wavelength of 490 nm was measured according to the manufacturer's instructions.

3. Results

3.1. Effect of 5′-NIO on KB cell proliferation and cytotoxicity

We previously reported three novel indirubin derivatives, 5′-nitro-indirubinoxime (5′-NIO), 5′-fluoro-indirubinoxime (5′-FIO) and 5′-trimethylacetamino-indirubinoxime (5′-TAIO) [17]. In order to assess the growth-inhibitory effect of the indirubin derivatives, we initially assessed the proliferation efficacy on KB oral carcinoma cells. As shown in Fig. 1A, high concentrations of indirubin derivatives (20 μM) inhibited the proliferation of KB cells. In particular, 5′-NIO showed the most potent growth-inhibitory effect among all the indirubin derivatives. Treatment with 2.5 μM of 5′-NIO reduced cell viability by approximately 81.5%. In order to evaluate the cytotoxic effect of 5′-NIO, we examined the lactate dehydrogenase (LDH) activity in 5′-NIO treated KB cells using a CytoTox96® Non-Radioactive Cytotoxicity Assay kit (Promega). As shown in Fig. 1B, treatment with 5 μM of 5′-NIO showed profound cellular toxicity in KB cells. As 5′-NIO induce the strong growth-inhibitory effect in KB cells,