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Tumor suppressor BLU promotes paclitaxel antitumor activity by inducing apoptosis through the down-regulation of Bcl-2 expression in tumorigenesis

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ABSTRACT

In this current work, we investigated whether BLU could enhance pro-apoptotic activity of chemotherapeutic drugs in ovarian carcinoma cells. A combination with a chemotherapeutic drug showed an additive effect, and this additive effect was supplemented by the enhancement of caspase-3 and -9 activities.

BLU and paclitaxel induced cell cycle arrest in the G2/M phase through the reduction of cyclin dependent kinase 1, cyclin B1, while promoting both p16 and p27 expression. In addition, both BLU and paclitaxel enhanced the expression of the pro-apoptotic protein Bax together with the suppression of anti-apoptotic protein Bcl-2, a protein which is well-known for its function as a regulator in protecting cells from apoptosis. As expected, the Bax and p21 activities were enhanced by BLU or paclitaxel, while a combination of BLU and paclitaxel were additively promoted, whereas Bcl-xL and NF-κB including Bcl-2 activity were inactivated. This study has yielded promising results, which evidence for the first time that BLU could suppress the growth of carcinoma cells. Furthermore, both BLU and paclitaxel inhibited the phosphorylation of signaling components downstream of phosphoinositide 3-kinase, such as 3-phosphoinositide-dependent protein kinase 1, and Akt. Also, BLU plus paclitaxel decreased phosphorylation of p70 ribosomal S6 kinase, as well as decreasing the phosphorylation of glycogen synthase kinase-3β, which is one of the representative targets of the mammalian target of rapamycin signaling cascade. These results provide evidence that BLU enhances G2/M cell cycle arrest and apoptotic cell death through the up-regulation of Bax, p21 and p53 expression.

1. Introduction

BLU tumor suppressor protein was frequently inactivated by being hypermethylated during tumorigenesis in a variety of primary malignancies such as cervical, ovarian, glioma, nasopharyngeal, and non-small cell lung carcinomas [1–5]. BLU has structural features of the protein involved in the C-terminal region, MYND domain, which are important transcriptional regulators of the signaling pathways. It consists of several cysteine and histidine amino acid residues, and is speculated to constitute a cellular protein–protein interaction. Recently, Dong et al. [6] reported that tumor suppressor BLU promotes pro-apoptotic activity of sMEK1 via physical cellular interaction. In addition, the N-terminal of BLU was observed directly its interaction with the C-terminal of sMEK1. The expression of the BLU protein was down-regulated in ovarian and cervical patients, and for these cases, was hypermethylated. However, it was found to be down-regulated in a population of lung, breast, kidney, neuroblastoma, and esophageal squamous cell carcinoma [1,2,4,7,8]. BLU is a stress-responsive gene, such as that of the heat shock family protein, and is regulated by E2F, while also showing both genetic and epigenetic abnormalities in tumors [2]. Epigenetic abnormalities are critical for the development of the tumor and in cell cycle progression for many tumor types. The BLU protein can functionally inhibit tumor formation *in vivo* [8].

Paclitaxel is widely used for the treatment of many cancers, including breast, advanced gynaecologic malignancies, head and neck, and non-small cell lung cancers, as an effective chemotherapeutic drug [9,10]. The anticancer drug paclitaxel binds to α/β-tubulin, inhibiting the disassembly of microtubules [11] and interfering with the cell cycle, while also inducing programmed cell death [12]. In the case of tumors, chemotherapy can generally be used in addition to other technologies, such as surgery and radiation therapy.

In present study, we investigated whether BLU can exert therapeutic agents to control the function of tumor growth in carcinoma cells. However, the effects of BLU on apoptosis and its underlying

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biological mechanism have not been well understood. This study was initially designed to explore the major role in regards to the suppression of the PI3k/Akt signaling regulators and the susceptibility to the effects of BLU alone or combined with paclitaxel in ovarian carcinoma cells. Also, we found that BLU enhanced apoptosis by arresting the PI3K/Akt- and mTOR-dependent cell cycle, and by inhibiting the Bcl-2 family-mediated cell proliferation pathway. Herein, the data we obtained provides direct evidence that BLU is one of the critical modulators of paclitaxel-stimulated cell death in human carcinoma cells, which could be a molecular basis for future planned pre-clinical and clinical trials of BLU in ovarian cancer.

2. Materials and methods

2.1. Chemicals, reagents, and antibodies

z-DEVD-fmk and z-LEHD-fmk inhibitor were obtained from Sigma (St. Louis, MO). Other anti-cancer drugs and chemicals were also purchased from Sigma. The primary antibodies used in this study were anti-BLU, anti-pro-caspase-3, anti-cyclin B1, anti-CDK1, anti-Bcl-2, anti-Bcl-xL, anti-Bax, anti-p53, anti-Akt, anti-phospho-Akt, anti-PI3K, anti-phospho-PI3K, anti-PDK-1, and anti-phospho-PDK-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p16, anti-p21, anti-p27, and anti-NF-κB (Oncogene, San Diego, CA), anti-mTOR, anti-phospho-mTOR, anti-p70S6K, anti-phospho-p70S6K, and anti-GSK-3β, and anti-phospho-GSK-3β (BDPhamingen, San Diego, CA), anti-PARP (BD Biosciences, Diego, CA), and β-actin (Sigma).

2.2. Substrate-based caspase-3 and -9 activity analysis

Caspase activity was assessed as previously reported [13]. In brief, 2.5×10^5 cells were seeded for 24 h, the cells were collected by centrifugation for 25 min at 12,000*g* at temperature of 4 °C, and the pellets were suspended in lysis buffer, then incubated at 37 °C for 1 h with specific actyl-DEVD-7-amino-4-trifluoromethyl coumarin (caspase-3) or actyl-LEHD-7-amino-4-trifluoromethyl coumarin (caspase-9) as the substrate, according to the manufacturer's instructions (Promega, Medison, WI).

For analysis of PARP cleavage, we performed the procedures as described in the previous study [14]. Briefly, 30 μg of protein were added with 60 μM biotinylated NAD in a 30 μl final volume of PARP reaction buffer (50 mM Tris–HCl, pH 8.0 and 25 mM $MgCl_2$) for 1 h at 37 °C.

2.3. Cell cycle analysis and annexin V staining

Cell cycle distributions in cells were measured with propidium iodide (PI) staining as previously described [15]. Apoptosis was calculated by staining with fluorescein isothiocyanate (FITC)-labeled annexin V. In brief, cells transfected/treated with control (expression vector only), BLU, paclitaxel, or BLU plus paclitaxel were collected, washed with ice-cold PBS, and then resuspended with binding buffer. After incubation for 1 h at 37 °C, the cells were treated with fluorescein isothiocyanate (FITC)-labeled Annexin V for 15 min, according to the manufacturer's directions (Boehringer Mannheim, Mannhein), and then the data of samples were measured with a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ).

2.4. Luciferase reporter assay

Luciferase activity was measured using a dual luciferase reporter assay system (Promega, Madison, WI). In brief, cells at 85% confluency were transiently transfected with each indicated reporter plasmid. After lysis, lysates were cleared with centrifugation at 14,000 rpm for 15 min and cell extracts were incubated with the luciferase substrate reagent at room temperature for 30 min. Then, a 5 μl aliquot of each sample was placed into the MicroLumat Plus LB96V luminometer.

2.5. Statistical analysis

All data values are represented as the mean ± SD and analyzed using the Student's *t*-test. Significant differences of 95% confidence ($P < 0.05$) are depicted with an asterisk (∗) on each graph.

3. Results

3.1. BLU ectopic expression suppresses carcinoma cell growth and additively controls with a chemotherapeutic drug

To explore the cytotoxic effects of BLU during paclitaxel-treated cell cycle arrest, exponentially growing cells were transfected/treated with various concentrations of BLU or paclitaxel chemotherapeutic anti-cancer drug. As expected, there was cell death effect in a dose-dependent manner. The maximum values of cell death were about 0.6–0.7 μg for BLU and 20–21 μM for paclitaxel (Fig. 1A). In order to confirm this possibility, we transfected/treated OVCAR-3 carcinoma cells with BLU (0.6 μg) and paclitaxel (20 μM) and determined cell viability, respectively. In the case of BLU, cells were suppressed to approximately 40% when compared to cells transfected with the control (expression vector only). Treatment with paclitaxel decreased cell viability by 45% and BLU plus paclitaxel was additively inhibited (Fig. 1B, *left* panel). In addition, we analyzed the possible additive or synergistic effects of BLU in combination with paclitaxel by flow cytometry. As shown in Fig. 1B (*right* panel), co-treatment with BLU and paclitaxel had an additive effect, suggesting that BLU coupled with paclitaxel additively inhibits cell growth.

Subsequently, we measured whether this effect of BLU and paclitaxel were related with the activation of caspase-3 and -9. The level of caspase-3 and -9 activities were activated in BLU-transfected cells as well as in paclitaxel-treated cells, while also being highly activated in BLU plus paclitaxel treatment when compared with the levels of the singly treated cells, as well as with the control (Fig. 2A and B). In western blotting, all types of BLU, paclitaxel, or BLU plus paclitaxel markedly promoted the cleavage of caspase-3. Specifically, in paclitaxel and BLU plus paclitaxel, the degree of caspase-3 cleavage was more induced than a single transfectant of BLU (Fig. 2C). Caspase-3 and -9 plays a major role as a critical regulator in cell death progression [16]. Also, the cleavage of PARP was highly activated in combination of BLU plus paclitaxel (Fig. 2D). Next, to validate the effects of caspase-3 and -9 inhibitors on cell death stimulated by BLU, paclitaxel, or a combination treatment, were used to treat OVCAR-3 cells with either z-DEVD-fmk or z-LEHD-fmk, which are specific inhibitors of caspase-3 and -9, respectively, for 3 h before transient BLU transfection, after which the cells were grown for 24 h. Treatment with z-DEVD-fmk or z-LEHD-fmk remarkably protected the BLU-transfected cells from apoptotic death. Similar results were observed when the cells were treated with paclitaxel as well as combined of BLU plus paclitaxel (Fig. 2E and F). Thus, BLU over-expression appears to be capable of additively suppressing cell growth by inducing cell death in carcinoma cells.

3.2. Cell cycle progression and expression of cell cycle-related proteins in carcinoma cells

To further clarify the biological functions of BLU or paclitaxel-stimulated cell death, the expression levels of cell cycle-related