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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

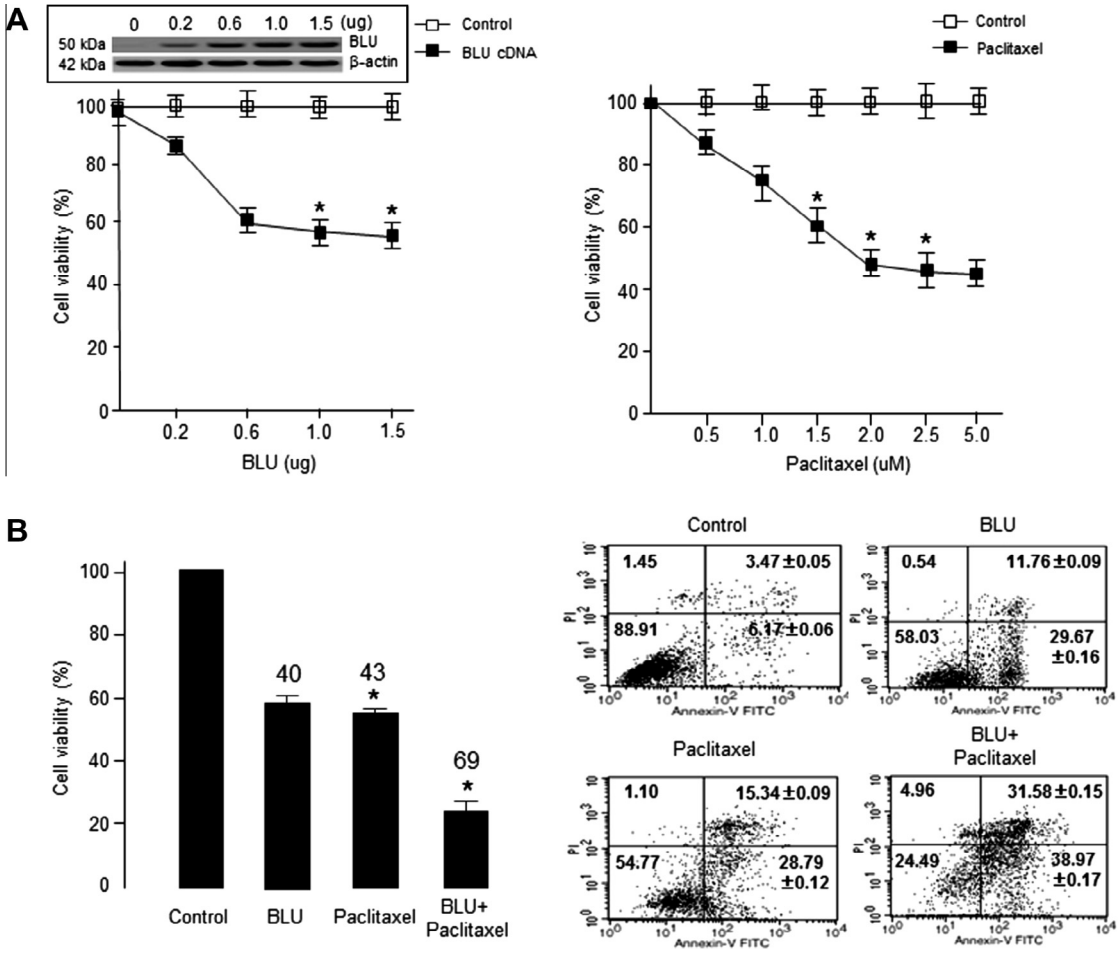

Fig. 1. Growth-inhibitory effects of BLU and paclitaxel in OVCAR-3 carcinoma cells *in vitro*. (A) Exponentially-growing cells were transfected/treated with the indicated concentrations of BLU or paclitaxel. Growth inhibitory effect was measured using MTT assay. (B) Cells transfected/treated by BLU, paclitaxel, or BLU plus paclitacxel, respectively. All of the floating and adherent cells were collected, and then the cells were determined by propidium iodide staining. Significant differences of 95% confidence ($P < 0.05$) are depicted with an asterisk (*) for each graph.

protein were investigated in OVCAR-3 ovarian carcinoma cells and then validated using an annexin/propidium iodide-based FACS analysis. p16, p27, cyclin-B1, and CDK1 are well-known as critical regulators of cell cycle progression and arrest. The expression levels of cyclin B1 and CDK1, which are associated with the transition of the G_2 to M phase, was greatly reduced, whereas the CDK inhibitors p16 and p27, which are associated with the disturbance of cell cycle progression in the G_2/M phase, were enhanced (Supplementary Fig. 1B). As shown, this displayed a significantly decreased expression of cyclin B1 and an enhanced level of p27, which is consistent with the G_2 phase arrest of cell cycle progression (Supplementary Fig. 1A, B). Our findings suggest that BLU and paclitaxel suppresses cell growth by inducing G_2 phase arrest in carcinoma cells.

3.3. BLU and paclitaxel controls the apoptosis-regulatory proteins, and regulates Bcl-2 family and NF-κB activity

p53 and NF-κB molecules are well-known as key mediators in the signaling pathways of cell viability and cell death [17,18], and are also known as pivotal regulators of the Bcl-2 family proteins [19–21]. To further gain evidence for whether there were any effects on the expression of BLU or paclitaxel-stimulated cell death, p21 was found to be transcriptional target of p53, so the expression of p53, including the Bcl-2 family member proteins, were investigated (Fig. 3A). The expression levels of the p53 protein were remarkably promoted after the ectopic expression of either BLU or paclitaxel-treated cells, along with Bax and p21. In contrast, expression of the anti-apoptotic proteins Bcl-xL, Bcl-2, and NF-κB were dramatically reduced. In addition, the promoter activities of Bax and p21 were up-regulated, whereas Bcl-xL and NF-κB activities were significantly down-regulated by BLU, paclitaxel, and BLU plus paclitaxel. Thus, it can be postulated that either BLU or paclitaxel promotes G_2 cell cycle arrest by inducing transcription of p21 (Fig. 3B and C).

3.4. Physically interaction of BLU with Bcl-2 family such as Bcl-2 and Bcl-xL

To understand the molecular biological mechanism involved in the induction of apoptosis by BLU, we used a yeast two-hybrid system and co-immunoprecipitation assay. We first assessed the binding activity of BLU on Bcl-2 family in the cells. Positive interaction was monitored by using both cell growth with leucin-deficient plate and β-galactosidase activity with ONPG. An empty construction (vector only) was subjected as the negative control. As presented in Fig. 4A, the β-galactosidase activity between BLU and Bcl-2 as well as Bcl-xL was fully activated (97.13 ± 1.36 and 89.97 ± 1.29), but not with empty vector (vector only). To further confirm the direct interaction between BLU and BCl-2 family that we introduced in the yeast two-hybrid assay, we investigated by co-immunoprecipitation. Gene constructs of BLU (pcDNA3.1/Flag-

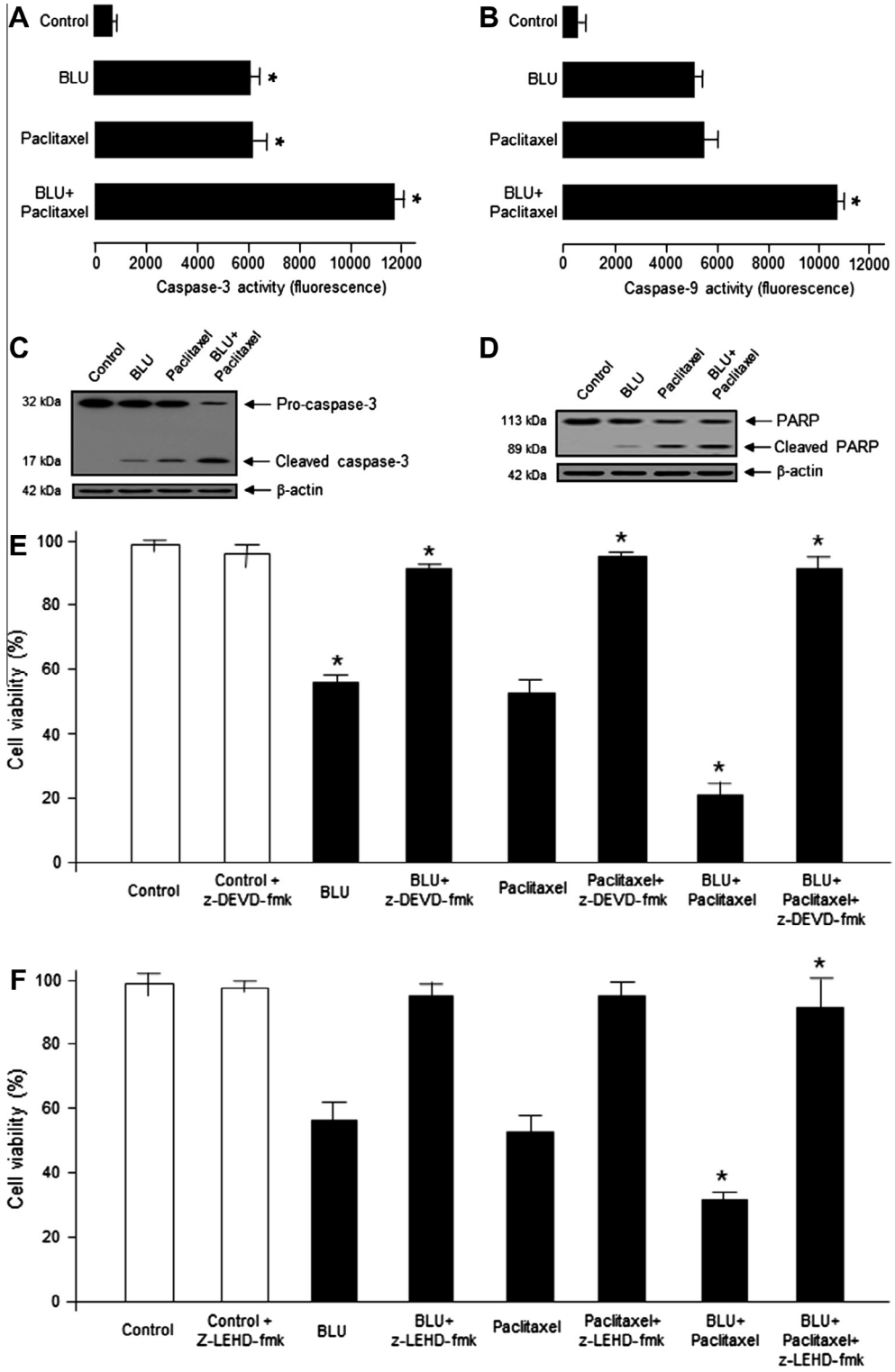

Fig. 2. The additive apoptotic effects of BLU and paclitaxel as a traditional chemotherapeutic drug. (A and B) Caspase-3 and -9 activities after transfection/treatment with BLU or paclitaxel were calculated using actyl-DEVD-7-amino-4-trifluoromethyl or actyl-LEHD-7-amino-4-trifluoromethyl coumarin as the substrate. (C and D) Caspase-3 and PARP cleavages enhanced by BLU, paclitaxel, or BLU plus paclitaxel transfection/treatments. Samples of protein extracts were resolved by SDS–PAGE gel, and immunoblotted with the indicated antibodies against caspase-3 and PARP. (E and F) The caspase-3- and -9-specific inhibitors z-DEVD-fmk and z-LEHD-fmk, respectively, were introduced to control-treated cells or cells treated with BLU, paclitaxel, or BLU together with one of the other drugs. The cells were then stained with trypan blue and cell viability was determined.

BLU) and Bcl-2 or Bcl-xL (pcDNA3.1-Bcl-2 or Bcl-xL), or pcDNA3.1/Flag-BLU and vector only (pcDNA3.1) were co-transfected into HEK293T cells. Subsequently, an immunoprecipitation was performed by using anti-Flag antibody with lysates from both transfected cells. After immunoprecipitation, the precipitated proteins were immunoblotted using indicated anti-BLU, anti-Bcl-2 or anti-Bcl-xL antibody, respectively. In the Fig. 4B, both pcDNA3.1-Bcl-2 and Bcl-xL were co-immunoprecipitated with pcDNA3.1/Flag-BLU

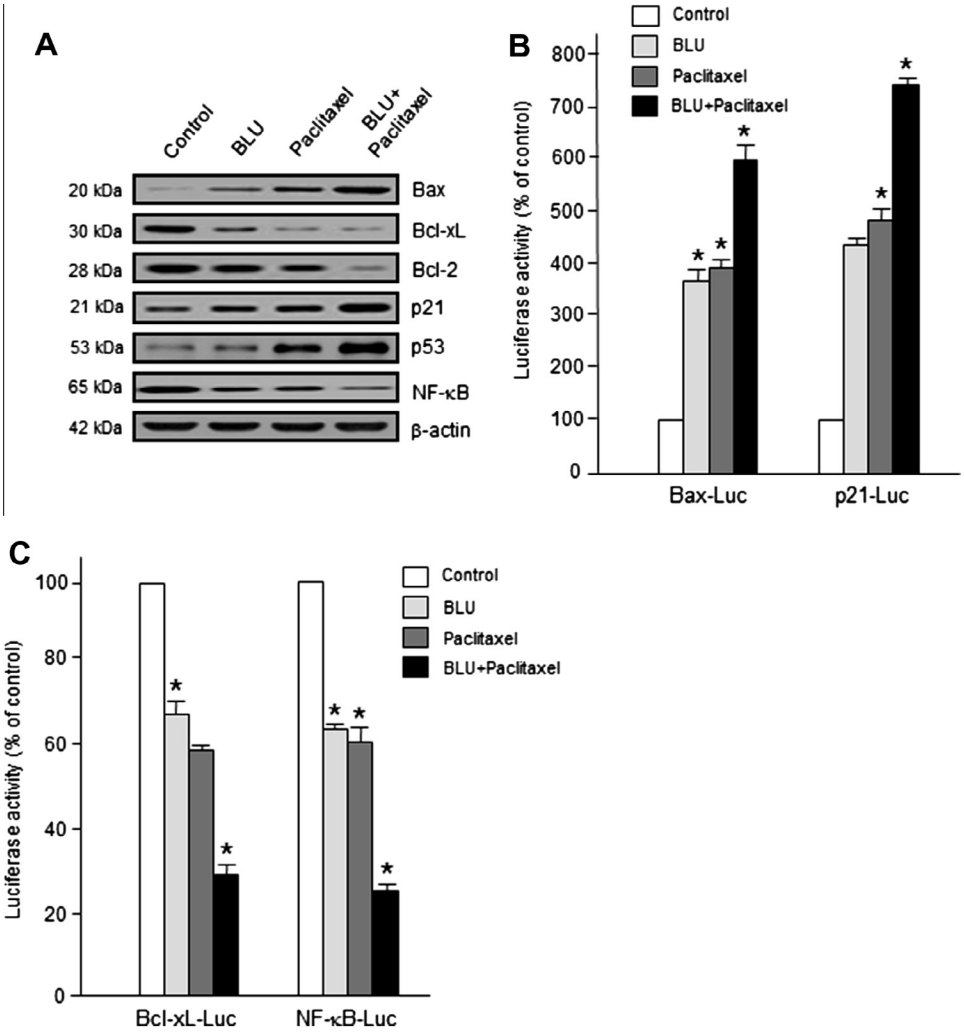

Fig. 3. The expression levels of BLU or paclitaxel on the apoptosis-related proteins, and transcription activity of Bax, p21, Bcl-xL, and NF-κB. (A) OVCAR-3 carcinoma cells were transfected/treated with the control (expression vector only), BLU, paclitaxel, or BLU plus paclitaxel, respectively. Then, the protein expression of the apoptosis-regulatory genes, which include Bax, Bcl-xL, Bcl-2, and p53 were tested. (B and C) Promoter activities of Bax, p21, Bcl-xL, or NF-κB were measured by the reporter assay with Bax (Bax-Luc), p21 (p21-Luc), Bcl-xL (Bcl-xL-Luc), or NF-κB (NF-κB-Luc), respectively. The values shown represent the mean ± SD. $^{*}P < 0.05$.

(*lane 2* in *upper* panel), whereas not with pcDNA3.1 (vector only) (*lane 1* in *upper* panel). We then examined the interaction between endogenous BLU and Bcl-2 family. Tumor suppressor BLU is participated to interact with the Bcl-2 or Bcl-xL of Bcl-2 family (Fig. 4C). To explore whether how BLU regulates Bcl-2, the effect of BLU on the Bcl-2 transcription was also assessed by a luciferase reporter assay, using a construct introducing Bcl-2 promoter fused to the luciferase gene. The luciferase activity was suppressed by the transfection of BLU in a dose-dependent manner (Fig. 4D), further providing the importance of BLU for the control of Bcl-2 activity. Our results indicate that ectopic expression of BLU inhibits its transcriptional activity. Next, we checked the effect of BLU on Bax expression in OVCAR-3 carcinoma cells. The over-expression of BLU significantly activated Bax expression, whereas siBLU had no affect (Fig. 4E). Collectively, our results strongly suggested that BLU directly interacts with Bcl-2 family in cellular physiological condition.

3.5. Interruption of the phosphorylation of Akt signaling components by BLU and paclitaxel

Akt, as a pivotal downstream regulator of PI3K, activates mTOR through a variety of biological mechanisms, including phosphorylation and the inactivation of apoptosis-associated proteins [22,23]. To explore the mechanism underlying its effects, apoptosis was induced by BLU, paclitaxel, or BLU plus paclitaxel. We investigated the involvement of PI3K, PDK-1, mTOR, p70S6K, and GSK-3β, an up- and down-stream target regulator of Akt, in this pathway. As expected, BLU, including paclitaxel and BLU plus paclitaxel, successfully suppressed the phosphorylation of PI3K, as well as the phosphrylation of PDK-1 and Akt (Supplementary Fig. 2). In addition, transfection/treatment with BLU or paclitaxel markedly reduced the level of phosphorylated p70S6K, as well as the level of phosphorylated GSK-3β, members of the best characterized targets of the mTOR complex. Taken together, BLU and paclitaxel suppressed the growth of carcinoma cells through the mTOR pathways-dependent on apoptosis, as well as promoting G_2 phase arrest of cell cycle progression in ovarian tumorigenesis.

4. Discussion

Generally, human solid malignancies are the leading cause, through various forms of numerous epigenetic alterations during the biological processes. Epigenetic changes are a well-established condition that plays a pivotal role in the embryonic development and progression of cancer. Epigenetic molecular mechanisms are

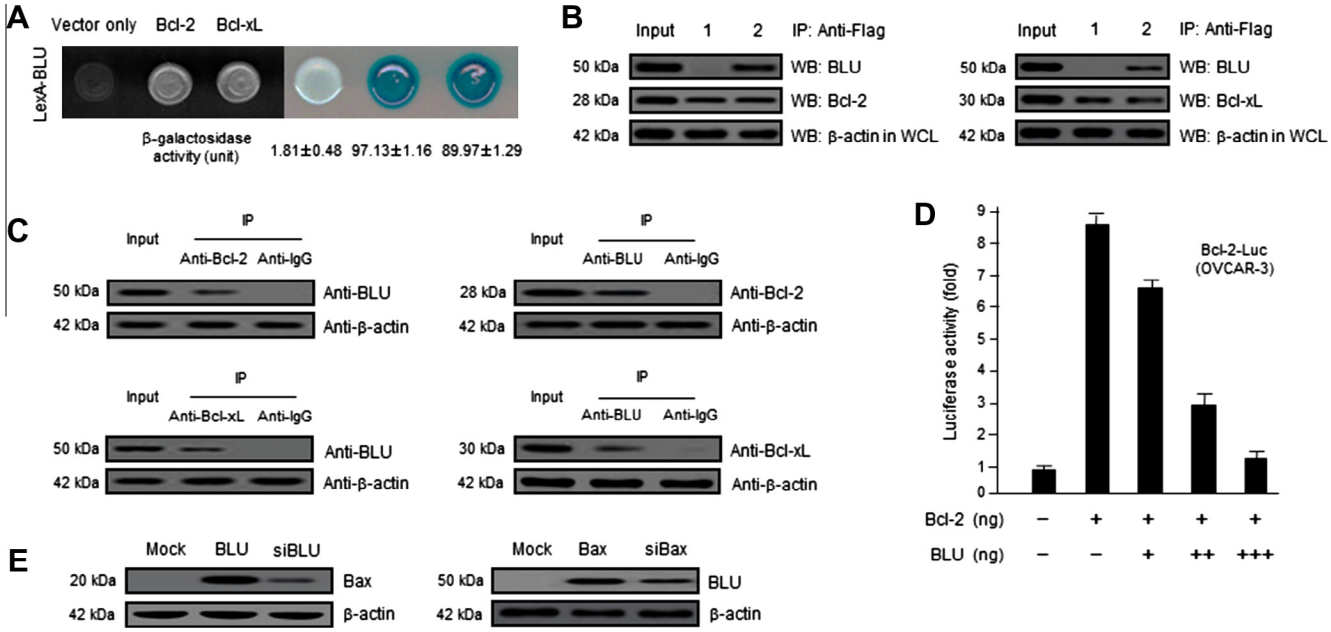

Fig. 4. Physical interaction between BLU and Bcl-2 family. (A) Positive interactions were investigated by observing cell growth on medium lacking leucine, and by the formation of a blue colony on the X-gal plate. (B) Co-immunoprecipitation of BLU with Bcl-2 and Bcl-xL of Bcl-2 family. Immunoprecipitation was performed using an anti-Flag antibody with lysates from both transfected HEK293T cells. *Lane 1*, pcDNA3.1 (vector only) and pcDNA3.1/Flag-BLU transfectant; *lane 2*, pcDNA3.1/Flag-BLU and pcDNA3.1-Bcl-2 or Bcl-xL transfectant. (C) Co-immunoprecipitation between the endogenous BLU and Bcl-2 family display the interactions of the two proteins. (D) Inhibition of Bcl-2-dependent transcription by BLU. OVCAR-3 carcinoma cells were co-transfected with 500 ng of Bcl-2-Luc, 500 ng of a Bcl-2 expression plasmid (pcDNA3.1/Bcl-2), and increasing concentrations of plasmid-encoding BLU (pcDNA3.1/Flag-BLU) (50, 250, and 500 ng). (E) To address whether BLU is specifically required for the activation of Bax, we transfected with BLU or siBLU in OVCAR-3 carcinoma cells, then compared the cellular levels of Bax by immunoblotting.

heritable and reversible with DNA methylation and histone modifications [24,25]. Our recent findings indicate that BLU loses its expression in ovarian and cervical carcinoma due to promoter hypermethylation [6,26]. However, the underlying molecular mechanisms undertaken by BLU in suppressing carcinoma cell proliferation are not fully understood. In this present study, using a carcinoma *in vitro* model system, we found a new crucial molecular mechanism of BLU as a novel effective regulatory protein that can target the Akt/mTOR signaling pathway. Ectopic expression of BLU significantly suppresses cell proliferation by inducing apoptosis, increased pro-apoptotic Bax protein expression, and decreased Bcl-xL expression as well as anti-apoptotic Bcl-2 protein in OVCAR-3 carcinoma cells. The p21 protein was highly up-regulated in BLU-transfecting cells, while NF-κB protein expression was significantly down-regulated, which may provide a situation for cell death in carcinoma cells (Fig. 3).

Most of the previous studies have shown only the methylation of BLU in primary cancers, such as gliomas, nasopharyngeal carcinoma, neuroblastomas, and cervical carcinoma [1,2,4,5]. In some tumors, hypermethylation is observed during the early stages of glioma and the cervical squamous cell [1,5]. In the BLU mechanism studies, Qiu et al. [2] found that BLU can be activated by environmental stress factors such as heat shock, and is regulated by E2F in nasopharyngeal malignancies.

The biological mechanism of the growth-inhibitory effect of the BLU protein in carcinoma cells was observed to be associated to the induction of cell cycle arrest in the G_2 phase (Supplementary Fig. 1A). Also, the expression levels of cyclin B1 and CDK1, which are related to the transition of the G_2 to M phase, were greatly decreased, whereas the CDK inhibitors, p16 and p27, which are associated with the disturbance of cell cycle progression in the G_2/M phase, were activated (Supplementary Fig. 1B). Our data results suggest that BLU expression may be frequently associated with human cancer progression from methylation studies. However, the biological role of BLU, in chemotherapeutic drug resistance has not been established. Herein, we also monitored that the change in BLU expression is associated with paclitaxel sensitivity. A combination with paclitaxel highly up-regulated the expression of Bax and p21 in OVCAR-3 carcinoma cells (Fig. 3A). Therefore, these results strongly imply that BLU additively suppresses cell proliferation through p21.

The PI3K/Akt signaling pathway is frequently activated in various human malignancies, and plays an important role in the essential cellular functions, such as tumor growth, migration, differentiation, and resistance to apoptotic induction in tumorigenesis. Thus, the down-regulating and targeting of PI3K or Akt activity is essential for tumor therapy [27]. Paclitaxel is mostly responsible for promoting cell cycle arrest and cell death in various types of carcinoma cells. Torres et al. [28] reported that the dose-dependent effects of paclitaxel on the cell cycle validated cell accumulation in the G_2/M phase and apoptosis. Importantly, paclitaxel and some other chemotherapeutic agents inactivate Akt, thus causing or enhancing apoptosis leading to the reduced survival of cancer cells [29]. In our present study, we have integrated the biological role among these molecules in ovarian carcinoma. Accordingly, it is thought that BLU plus paclitaxel-stimulated G_2/M phase cell cycle progression might be caused by the down-regulation of the G_2 phase-related kinases such as cyclin B1 and CDK1 (Supplementary Fig. 1B). In addition, the level of caspase-3 and -9 activities were activated by BLU or paclitaxel-treated cells, and PARP cleavage was also activated (Fig. 2A–D). Meanwhile, BLU additively reduced paclitaxel-induced phosphorylation of essential components in the PI3K/Akt signaling pathways, such as PDK-1, mTOR, p70S6K, and GSK-3β (Supplementary Fig. 2). Our findings indicate that BLU and paclitaxel inhibits cell growth by inducing G_2 phase arrest in carcinoma cells.

In summary, this study systematically analyzed the cytotoxic effects using an *in vitro* model system with the tumor suppressor BLU protein and paclitaxel, a traditional chemotherapeutic drug in tumorigenesis. Both BLU and paclitaxel treatment inhibited

the cell growth of OVCAR-3 carcinoma cells through caspase-dependent apoptosis as well as cell cycle arrest by inducing the transcription of p21. Importantly, our results suggest the first findings on the molecular pro-apoptotic mechanisms of BLU and paclitaxel in human ovarian tumorigenesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.061.

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