UNBS5162 inhibits the proliferation of esophageal cancer squamous cells via the PI3K/AKT signaling pathway

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Abstract. C-X-C motif chemokine ligand (CXCL) signaling has been demonstrated to be involved in cancer invasion and migration; therefore, CXCL antagonists may serve as anti-cancer drugs by preventing tumor proliferation. The present study aimed to investigate whether a pan antagonist of CXCLs, UNBS5162, may inhibit esophageal cancer proliferation and to identify the underlying mechanisms. Cell proliferation and cell colony formation results, which were determined by a Cell Counting Kit-8 assay and crystal violet staining, respectively, demonstrated that UNBS5162 inhibited esophageal cancer cell proliferation. Following treatment with UNBS5162, Transwell migration and Matrigel invasion assays, and flow cytometry with Annexin V-fluorescein isothiocyanate and propidium iodide staining, were performed to investigate cell migration, invasion and apoptosis in human esophageal cancer cells. The results indicated that invasion and migration was reduced in UNBS5162-treated cells, while apoptosis was increased. Western blotting experiments confirmed that UNBS5162 downregulated the protein expression of proteins associated with the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, including the levels of phosphorylated (p)-AKT, p-mechanistic target of rapamycin kinase, ribosomal protein S6 kinase β1 and cyclin D1. In addition, upregulated expression of programed cell death 4 was observed following UNBS5162 treatment. The present study demonstrated that UNBS5162 is a novel naphthalimide that may have potential therapeutic use for the prevention of esophageal cancer proliferation and metastasis via the PI3K/AKT signaling pathway.

Introduction

Combined, digestive tract tumors are currently the most common group of tumor types, and the incidence and mortality accounts for ~30% of all tumors worldwide (1). Esophageal cancer, as one of the most common digestive tract malignancies, is the 6th leading cause of cancer-associated mortality globally (2). Two pathological types of esophageal cancer exist, which are esophageal adenocarcinoma and esophageal squamous cell carcinoma. Geographical distribution differences in the incidence of esophageal cancer are observed; in western countries, the incidence of esophageal adenocarcinoma is predominant, while in East Asia the incidence of esophageal squamous cell carcinoma predominates (3). Despite advanced treatment with concurrent chemotherapy alone or as an adjunct to radiotherapy or surgery, the 5-year survival rate is <20% for patients with esophageal cancer (4). Thus, the development of chemotherapy drugs with enhanced efficacies is urgently required for esophageal cancer.

Chemokines consist of a family of ~50 low-molecular-weight chemotactic cytokines (5). The chemokine family is divided into four separate subtypes, including C-X-C (CXC), C-C (CC), C-X3-C (CX3C) and X-C (XC) motif chemokine ligands, where X represents any amino acid positioning of the conserved cysteines in the aminoterminal region of these proteins (6). At present, the CXC motif chemokine ligands (CXCLs) that have been identified are termed CXCL1-CXCL17 (7,8). Chemokines have indirect roles in tumor development by affecting angiogenesis and tumor-leukocyte interactions, and also have direct effects on tumor transformation, survival and growth, and invasion and metastasis. Various studies have reported that CXCLs are overexpressed in numerous tumor types, including esophageal cancer. Bruyère et al (9) revealed that the proangiogenic CXCL1, CXCL2, CXCL8, CXCL16 and CXC motif receptor (CXCR) 4 were markedly overexpressed in esophageal cancer cells. Furthermore, CXCL12 and its receptor, CXCR4, are reported to be implicated in the metastasis of esophageal squamous cell carcinoma to lymph nodes (10). Notably, Wang et al (11) reported that CXCL1-CXCR2 and CXCL2-CXCR2 signaling is associated with increased esophageal cancer cell proliferation and may therefore be implicated in the development of esophageal tumors. Chemokine antagonists prevent the binding of chemokines and chemokine receptors, and thus have therapeutic effects in various tumor
types. Based on these previous observations, the present study aimed to identify an efficient chemokine antagonist as a potential effective chemotherapy drug for human esophageal cancer.

UNBS5162 is a pan antagonist of CXCLs and is also a naphthalimide. As an antagonist of CXCL, to the best of our knowledge, the effect of UNBS5162 in esophageal squamous cell carcinoma has not previously been reported. Furthermore, as a naphthalimide, the effect of UNBS5162 in other tumor types is scarcely reported, including esophageal cancer. Therefore, the present study aimed to investigate the effect of UNBS5162 on the proliferation and metastasis of esophageal squamous cell carcinoma, in addition to the underlying mechanisms of UNBS5162-induced antitumor effects.

Materials and methods

Cell culture. The KYSE-30 human esophageal squamous cell carcinoma cell line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with an atmosphere of 5% CO₂ in a humidified cell incubator. Cell viability was detected every 24 h for a total of 72 h. Prior to detection, 10 µM UNBS5162 (Medchem Express, South Brunswick, NJ, USA) was added to cells. The negative control (NC) group was treated with dimethyl sulfoxide (DMSO; 1:1,000; Amresco, Solon, OH, USA) instead.

Cell suspension assay. Cell suspension (100 µl) was routinely seeded into 96-well plates (1x10⁴ cells/well). Cells were treated with 10 µM UNBS5162 or DMSO (1:1,000) which were incubated at 37°C with an atmosphere of 5% CO₂ in a humidified cell incubator. Cell viability was detected every 24 h for a total of 72 h. Prior to detection, 10 µl Cell Counting Kit-8 (CCK-8) reagent (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) was added to cells and incubated at 37°C for 90 min, subsequently, the optical density value at 450 nm was detected and a proliferation curve was plotted.

Cell colony formation. Cells suspensions containing 500 cells were cultured in 60 mm dishes containing 5 ml prewarmed culture medium at 37°C. Dishes were gently rotated to disperse the cells homogeneously. Cells were treated with 10 µM UNBS5162 for 48 h at 37°C in an atmosphere of 5% CO₂ and 95% air. Following treatment, media was removed and cells were washed twice with PBS. Then cells were maintained in DMEM with 10% FBS for 1-2 weeks at 37°C in a humidified cell incubator with an atmosphere of 5% CO₂. When visible clones were observed, culture was terminated, the supernatant was discarded and cells were washed twice with PBS. Cells were subsequently fixed with 4% paraformaldehyde for 30 min at room temperature, followed by gentle washing with PBS three times and staining with 0.1% crystal violet for 30 min at room temperature. Cells were washed with running water and processed by air drying. Visible colonies consisting of ≥50 cells were counted and the size and number of clones were compared.

Invasion and migration assays. Cancer cell invasion assays were performed using 24-well Transwell plates. Matrigel matrix gel (100 µl; BD Biosciences, Franklin Lakes, NJ, USA) was diluted with serum-free DMEM overnight at 1:6 and was added to the upper chamber of the 24-well plate in the Transwell chamber. Following treatment of cells with 10 µM UNBS5162 for 48 h at 37°C with an atmosphere of 5% CO₂ in a humidified cell incubator, 1x10⁵ cells were seeded into the upper chambers resuspended in 100 µl serum-free DMEM. DMEM (500 µl) with 10% PBS was added to lower chambers. Following incubation at 37°C overnight, non-invading cells in the upper chambers were removed by scrubbing gently using a cotton-tipped swab. The cells that invaded the lower chambers were fixed in 4% paraformaldehyde for 30 min at room temperature and stained with 0.1% crystal violet for 20 min at room temperature. Following washing with PBS, the number of invading cells was determined by light microscope in five random fields for each insert (magnification, x100) and calculated as the mean number of cells per field.

The protocol for the migration assays was similar to the invasion assay, however, Matrigel matrix was not added to the upper chamber of Transwell inserts and the number of cells seeded in the upper chambers was 5x10³.

Flow cytometry analysis. Following treatment of cells with 10 µM UNBS5162 for 48 h at 37°C, the medium was removed and replaced with serum-free DMEM. The cells were starved for 24 h under normal conditions. Cells were digested by trypsin without EDTA, collected into a centrifuge tube and centrifuged at 800 x g at 4°C. Cells were resuspended in PBS that was precooled at 4°C, centrifuged at 800 x g for 5 min and the supernatant was carefully aspirated. Subsequently, 1X binding buffer diluted by 4X binding buffer [Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit; both from Beijing 4A Biotech, Co., Ltd., Beijing, China] was used to resuspend the cells at a density of 1-5x10⁶ cells/ml at 4°C. Cell suspension (100 µl) and Annexin V-FITC (5 µl; Annexin V-FITC/PI kit; Beijing 4A Biotech, Co., Ltd.) were added to a 5 ml flow tube and, following mixing, incubated at room temperature in the dark for 5 min. Subsequently, 10 µl PI (Annexin V-FITC/PI kit; Beijing 4A Biotech, Co., Ltd.) was added for incubation at room temperature in the dark for 5 min and subsequently, 400 µl PBS was added for detection. Results were analyzed and processed with FlowJo software (version 7.6.3; FlowJo, LLC, Ashland, OR, USA).

Western blot analysis. After the NC and the experimental (10 µM UNBS5162) groups were treated for 48 h, 6-well plates were placed on ice and treated with radioimmunoprecipitation assay (RIPA) lysis buffer (CWBiotech Co., Ltd., Beijing, China) with the addition of protease inhibitors (Protease Inhibitor Cocktail; CWBiotech Co., Ltd.). Subsequently, the
concentration of extracted protein was measured by a BCA kit. Extracted proteins (20 µg) were mixed with loading buffer (5X; Thermo Fisher Scientific, Inc.) and boiled at 95˚C for 5 min. The mixture of denatured protein was separated by 10-20% polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and transferred to a polyvinylidene fluoride membrane (PVDF; Thermo Fisher Scientific, Inc.). Membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies against Bcl-2 (1:1,000, cat. no. 12789-1-AP), Bcl-2-associated X (Bax; 1:1,000, cat. no. 23931-1-AP), and cleaved-caspase-3 (1:1,000, cat. no. 25546-1-AP) (all from ProteinTech Group, Inc., Chicago, IL, USA), AKT (1:1,000, cat. no. 4691), phosphorylated (p)-AKT (1:1,000, cat. no. 4060), mechanistic target of rapamycin kinase (mTOR; 1:1,000, cat. no. 2983), and p-mTOR (1:1,000, cat. no. 5536) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) and ribosomal protein S6 kinase β1 (p70S6K, 1:1,000, cat. no. 14485-1-AP), programed cell death 4 (PDCD4; 1:3,000, cat. no. 12587-1-AP), cyclin D1 (1:5,000, cat. no. 60186-1-lg) and GAPDH (1:5,000, cat. no. 10494-1-AP) (all from ProteinTech Group, Inc.) overnight at 4˚C. Membranes were washed with PBS with Tween-20 (PBST) three times for 5 min and incubated with polyclonal horseradish peroxidase-conjugated goat anti-rabbit (1:10,000, cat. no. SA00001-2) and goat anti-mouse (1:10,000, cat. no. SA00001-1) (both from ProteinTech Group, Inc.) secondary antibodies for 1 h at room temperature. Following a final washing step, signals were developed by enhanced chemiluminescence reagent (PerkinElmer, Inc., Waltham, MA, USA) and quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.), with GAPDH as an internal control.

Statistical analysis. SPSS 18.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA) was used to analyze the experimental data. Data are presented as the mean ± standard deviation. Comparisons between the two groups were performed using Student’s t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

UNBS5162 inhibits the proliferation and colony formation of KYSE-30 squamous cell esophageal carcinoma cells. To investigate the effect of UNBS5162 on cell growth, the proliferation of KYSE-30 cells treated with 10 µM UNBS5162 was investigated using a CCK-8 assay. As demonstrated in Fig. 1A, the proliferation of UNBS5162-treated KYSE-30 cells was significantly inhibited at 48 and 72 h, compared with the NC group (P<0.05). Furthermore, the inhibitory effects of UNBS5162 on the cell proliferation occurred in a time-dependent manner (Fig. 1A). To further investigate whether UNBS5162 has an effect on the survival of adherent cells and the number of formation of clones, a cell colony formation assay was performed. The results of the colony formation assay demonstrated that the ability of KYSE-30 cells to form colonies was significantly inhibited in the UNBS5162 group, compared with the NC group (P<0.05; Fig. 1B and C). The results presented
in Fig. 1 indicated that UNBS5162 was able to suppress the cell proliferation and the ability of squamous cell esophageal carcinoma cells to form colonies.

**UNBS5162 inhibits the migration and invasion of KYSE-30 squamous cell esophageal carcinoma cells.** As UNBS5162 inhibited the proliferation and colony formation of KYSE-30 cells, the effects of UNBS5162 on KYSE-30 cell migration and invasion were subsequently investigated. The results of the Matrigel invasion assay demonstrated that the invasion ability of KYSE-30 cells treated with UNBS5162 was significantly inhibited compared with the NC group (P<0.05; Fig. 2). In addition, the migration ability of KYSE-30 cells treated with UNBS5162 was also significantly inhibited compared with the NC group (P<0.05; Fig. 2). These results indicate that UNBS5162 significantly reduced the cell migration and invasion of KYSE-30 squamous cell esophageal carcinoma cells.

**UNBS5162 promotes the apoptosis of KYSE-30 squamous cell esophageal carcinoma cells.** Staining of KYSE-30 cells with Annexin V-FITC and PI, followed by flow cytometry, was performed to determine the proportion of apoptotic cells at 48 h after exposure to UNBS5162. Flow cytometry analysis demonstrated that the proportion of apoptotic cells were increased following treatment with UNBS5162 compared with the NC group (Fig. 3A). These results indicate that UNBS5162 may induce cell apoptosis. Various proteins participate in the apoptotic process. Therefore, to further confirm that UNBS5162 induces apoptosis in KYSE-30 cells, the protein expression of the proapoptotic proteins cleaved-caspase-3 and Bax, and the antiapoptotic protein Bcl-2, was detected in KYSE-30 cells by western blotting. The results demonstrated that the expression of Bax and cleaved-caspase-3 was increased, whereas the levels of Bcl-2 were decreased, in KYSE-30 cells treated with UNBS5162, compared with the NC group (P<0.05; Fig. 3B and C). Overall, these results indicate that UNBS5162 induced apoptosis in KYSE-30 squamous cell esophageal carcinoma cells.

**UNBS5162 inhibits the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway in KYSE-30 squamous cell esophageal carcinoma cells.** Previous studies have demonstrated that the activation of the PI3K/AKT signaling pathway may promote apoptosis. To determine the specific signaling pathway involved in UNBS5162-mediated apoptosis in KYSE-30 cells, the present study measured the activation of key signaling proteins, including AKT, mTOR, p70S6K, PDCD4 and cyclin D1 by western blotting. The results demonstrated that treatment with UNBS5162 exerted limited effects on AKT and mTOR expression, however, significant decreases in p-AKT, p-mTOR, p70S6K and cyclin D1 expression, and an increase in PDCD4 expression, were observed in the UNBS5162 group, compared with the NC group (P<0.05; Fig. 4). Therefore, UNBS5162 may inhibit the PI3K/AKT signaling pathway in KYSE-30 squamous cell esophageal carcinoma cells.
Chemokines were originally identified as chemotactic cytokines and were primarily thought to function in the migration of leukocytes during inflammation and immune surveillance (16). However, it has since been established that chemokines also exhibit various additional functions during physiological conditions, including roles in homeostasis, development, tissue repair and angiogenesis, and also during pathological conditions, including roles in autoimmune and inflammatory diseases (17). In addition, increasing evidence indicates that chemokines and their receptors have a strong influence on tumor development (18). Jin et al (19) reported that knockdown of CXCR6 in gastric cancer cells inhibited the cell proliferation, migration and invasion, and also reversed epithelial-mesenchymal transition (EMT). In addition, Xiang et al (20) demonstrated that cross-activation of CXCR4 and CXCR2 contributed to the migration and invasion, and EMT, of gastric cancer cells. Furthermore, CXCL5 was reported to promote osteosarcoma cell migration and invasion in autocrine- and paracrine-dependent manners (21). The use of chemokine receptor antagonists with natural ligands to chemokine receptors has received increased attention in research. In particular, the promotion of tumor cell proliferation and inhibition of tumor cell apoptosis by chemokines has been considered. For example, inhibition of CCL3 in vitro using α-CCL3 or evasin-1 (a CCL3-binding protein) impaired tumor cell invasion (22). Furthermore, targeted disruption of CXCL13 or its receptor, CXCR5, reduced the migration and tumorigenesis of prostate cancer cells (23). Consistently, the present study employed a CXCL antagonist, UNBS5162, which was demonstrated to inhibit squamous cell esophageal carcinoma cell proliferation, invasion and migration.

In addition to being an antagonist of CXCL, UNBS5162 is also a novel naphthalimide. Naphthalimides, which are DNA intercalators, have been investigated extensively as antitumor agents (24). Among antitumor agents, naphthalimide analog derivatives are among the most promising classes of anticancer drug candidates (25-27). Amonafide, elinafide and mitonafide have reached the clinical trials stage for the treatment of various tumors and exhibited excellent antitumor activity,
however, the majority were abandoned due to severe adverse effects such as dose-limiting or bone marrow toxicity (28). As a novel naphthalimide, UNBS5162 inhibition of tumor progression has only been reported once for prostate cancer; in vivo, UNBS5162 significantly decreased cell survival in PC-3 human prostate cancer cells and exhibited synergistic therapeutic effects when combined with paclitaxel (29). Notably, the present study, to the best of our knowledge, is first to demonstrate that UNBS5162, as a naphthalimide, inhibited esophageal cancer cell proliferation, invasion and migration. The effects of UNBS5162 in the present study are all based on results in the KYSE-30 esophageal cancer cell line, and the effects on additional esophageal cancer cell lines should be investigated in future experiments.

The present study also analyzed cell apoptosis and the associated mechanisms in esophageal cancer cells. UNBS5162 was demonstrated to induce cell apoptosis and inhibit the PI3K/AKT signaling pathway. Similarly, a previous study reported that CCR5 blockade was associated with a significant reduction in the migration of microglia, an effect that was mediated via the inhibition of the AKT pathway (30). The present study is the first to illustrate that inhibition of esophageal cancer cells using a CXCL antagonist may occur via inhibition of the PI3K/AKT signaling pathway. Previous studies have indicated that cell proliferation is strongly associated with cell apoptosis in the development of numerous types of cancer (31-35). In the present study, a reduction in cell proliferation was accompanied by increased apoptosis following treatment with UNBS5162, as determined by CCK-8 assays, flow cytometry and alterations in the expression of apoptosis-associated proteins. Caspase-3 is a marker for the induction of apoptosis (36). In the present study, UNBS5162 was demonstrated to activate cell apoptosis. As a result, levels of the antiapoptotic protein Bcl-2 were significantly downregulated, while levels of the proapoptotic protein Bax were increased, which led to caspase-3 upregulation. The results of western blotting indicated that cell apoptosis was stimulated by UNBS5162 treatment, which was consistent with a previous study (29), further confirming that UNBS5162 may exert its effects on tumor growth by apoptotic induction via the Bcl-2-regulated caspase-3 signaling pathway.

Furthermore, the results of the present study indicated that UNBS5162 significantly inhibited the PI3K/AKT signaling pathway in KYSE-30 esophageal cancer cells, as levels of p-AKT, p-mTOR, p70S6K and cyclin D1 were decreased, while increased PDCD4 expression was observed. It is established that the PI3K/AKT pathway regulates various cellular processes, including cell proliferation, apoptosis and tumorigenesis (37). The activation of AKT triggers cyclin D1 expression, which is implicated in the cell proliferation of various cancer cell types, including glioma cells (38). Therefore, inhibiting AKT activation and reducing cyclin D1 expression may suppress cancer cell proliferation. In addition, mTOR was reported to promote the proliferation of cancer cells via p70S6K stimulation (39). PI3K/AKT affect cell apoptosis through various mechanisms, including regulating the activity of Bcl-2 family members. Bcl-2 family members are divided into antiapoptotic (such as Bcl-2) and proapoptotic (such as Bax) proteins. PI3K-dependent activation of AKT promotes Bcl-2 expression and enhances antiapoptotic effects. At the same time, activation of the PI3K/AKT inactivates Bax, which further contributes to the inhibition of apoptosis (40). Furthermore, PI3K/AKT signaling also affects cell apoptosis by inhibiting caspase-induced cell apoptosis (41), and inhibition of glycogen synthase kinase 3 activity was reported to accelerate apoptosis (42). In addition, activation of AKT induced cell apoptosis through forkhead box O1-Fas ligand signaling (43), and inhibited the release of mitochondrial cytochrome c and proapoptosis factors, thereby inhibiting apoptosis (44). Consistent with the results of previous studies, the present study demonstrated that inhibition of AKT activity, accompanied by reduced Bcl-2 and enhanced Bax and caspase-3 levels, was associated with increased apoptosis in KYSE-30 esophageal squamous cell carcinoma cells. These results indicate that UNBS5162 may inhibit cell proliferation by suppressing the PI3K/AKT signaling pathway to induce apoptosis.

In conclusion, the present study characterized the anticancer properties and underlying mechanisms of UNBS5162. The results demonstrated that UNBS5162 is a promising anticancer agent for esophageal cancer and may be useful as an adjuvant. The anticancer effect of UNBS5162 via its naphthalimide properties has been illustrated, however, the potential candidate gene which has not been screened out requires substantial amounts of esophageal cancer samples, and further investigation regarding its interactome and the physiological cellular outcomes should be analyzed in future studies.

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References

hepatocellular carcinoma

Polyamine conjugates as antitumor agents

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Pathway loss for prostate tumorigenesis through the CXCL13-CXCR5


Balkwill FR: The chemokine system and cancer.

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