https://doi.org/10.31925/farmacia.2022.4.22 **ORIGINAL ARTICLE**

ANTITUMOR EFFECTS OF ANLOTINIB HYDROCHLORIDE ON HUMAN PANCREATIC CANCER CELLS

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Manuscript received: March 2022

Abstract

Anlotinib hydrochloride (AL3818), a new multi-kinase inhibitor, has shown promising antitumor efficiency in several advanced malignancies. However, data on AL3818 use for pancreatic cancer are still limited. This study aimed to investigate the antitumor activity of AL3818 and its underlying molecular mechanisms in human pancreatic cancer cells. The human pancreatic cancer cell line PANC-1 was exposed *in vitro* to increasing concentrations of AL3818 treatment (0.1, 0.5, 1, 2, or 5 μmol/L). The antiproliferative effect of AL3818 was determined by 3-(4 5-dimethyl-2-thiazolyl)-2 5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell cycle alterations and apoptosis rates were evaluated by flow cytometry, while expression of Toll-like receptor 4 (TLR-4), Nuclear factor kappa B (NF-κB), Vascular endothelial growth factor (VEGF), survivin, and Bax proteins were detected by Western blot. Our experimental data demonstrated a dose-dependent decrease in the proliferation of PANC-1 cells following treatment with AL3818. AL3818 efficiently induced G2/M phase cell cycle arrest and promoted cell apoptosis in a dose-dependent manner. In addition, expression levels of TLR-4, NF-κB, VEGF, and survivin proteins were decreased, while upregulation of Bax levels was achieved under AL3818 treatment. Our results suggested that AL3818 could interfere with PANC-1 cell proliferation and apoptosis *in vitro*. Treatment was also found to induce G2/M phase cell cycle arrest, possibly associated with upregulation of Bax protein and downregulation of VEGF, survivin, and TLR-4/NF-κB signalling pathway. Further research is clearly warranted to confirm these findings.

Rezumat

Clorhidratul de anlotinib (AL3818), un nou inhibitor de multi-kinază, a dovedit o eficiență antitumorală promițătoare în mai multe tumori maligne avansate. Acest studiu a avut ca scop investigarea activității antitumorale a AL3818 și a mecanismelor moleculare care stau la baza acesteia, în celulele cancerului pancreatic uman. Datele noastre experimentale au demonstrat o scădere doză-dependentă a proliferării celulelor PANC-1 în urma tratamentului. AL3818 a indus în mod eficient oprirea ciclului celular în faza G2/M și a determinat apoptoza celulară într-o manieră doză-dependentă. În plus, expresia proteinelor TLR-4, NF-κB, VEGF și a survivinelor a fost redusă, în timp ce nivelul Bax a crescut sub tratamentul cu AL3818. Rezultatele noastre sugerează că AL3818 ar putea interfera cu proliferarea și apoptoza celulelor PANC-1 *in vitro*. S-a constatat, de asemenea, că tratamentul a indus stoparea ciclului celular în faza G2/M, posibil asociat cu o creștere a proteinei Bax și o scădere a VEGF, a nivelului de survivină și a căii de semnalizare TLR-4/NF-κB.

Keywords: AL3818, multi-kinase inhibitor, PANC-1 cell line, proliferation, apoptosis, G2/M, TLR-4/NF-κB pathway, VEGF, Survivin, Bax

Introduction

In the area of personalized medicine, the management of advanced malignancies should be focused on molecularly targeted agents. Pancreatic cancer is the eleventh most common cancer in the world, with a higher incidence in industrialized countries and the USA, having a high mortality rate and a poor prognosis [1]. As the clinical symptomatology is silent in the early stage, the diagnosis is given in the advanced stage that cannot allow tumour resection [2].

Receptor tyrosine kinase (RTKs) is part of the family of transmembrane receptors that have shown to be associated with the development and progression of several diseases and cancers. In the last years, RTKs targeted by certain tyrosine kinase inhibitors proved their efficacy in the management of several cancers [3]. In this respect, anlotinib hydrochloride (AL3818), a new multi-targeted receptor tyrosine kinase inhibitor (TKI), has offered significant survival benefits in patients with non-small cell lung cancer or thyroid cancer [4, 5]. By inhibiting multiple growth factor receptors, such as the vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR) [6], and stem cell factor receptor c-Kit [7], AL3818 can exhibit both antitumor and anti-angiogenic activities [8, 9].

To date, AL3818 has been successfully utilized in the treatment of several cancer types, including colorectal [10], lung [11] and thyroid malignancies [12]. However, the role of AL3818 on pancreatic cancer has not yet been thoroughly explored. Therefore, this study was carried out to investigate the antiproliferative and

apoptotic effects of AL3818 and its underlying mechanisms of action in a PANC-1 cell line-based *in vitro* model of pancreatic cancer.

Materials and Methods

Cell line and culture conditions

The human pancreatic cancer cell line PANC-1 was purchased from Shanghai Aolu Biological Technology Co., Ltd, China. PANC-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), penicillin (1×10^5 U/L) and streptomycin (100 g/L) at 37°C in a 5% CO_2 atmosphere. All the chemicals and reagents were purchased from Shanghai Aolu Biological Technology Co., Ltd, China.

Morphological changes of PANC-1 cells

Morphological changes of PANC-1 cells following AL3818 treatment were assessed through haematoxylin and eosin (HE) staining and observed under a light microscope (Olympus Corporation, Japan).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay Six experimental cell groups were set up, with 6 replicates *per* group, and exposed to different concentrations of AL3818 (Shanghai Aolu Biological Technology Co., Ltd, China), *i.e.*, 0 µmol/L for the control and 0.1, 0.5, 1, 2 and 5 µmol/L in AL3818-treated groups. All cell groups were seeded in a culture medium until 80% confluence. Then, cells were collected after centrifugation (2000 rpm, 5 min) and counted using (the Neubauer) counting chamber. A total of 3×10^3 cells were placed in each well of a 96-well plate and cultured at 37°C, 5% CO_2, and saturated humidity until attachment. Then, six different concentrations (0, 0.1, 0.5, 1, 2, or 5 µmol/L) of AL3818 were added into each of the wells. Cell growth was monitored, and images were acquired every 24 h using a light microscope (Olympus Corporation, Japan). After 72 h of treatment, 20 µL of (5 mg/mL) MTT solution was added to each well, and the plate was further incubated for 4 h in a humidified atmosphere, followed by the addition of 150 µL Dimethyl sulfoxide (DMSO) to dissolve the coloured crystals. The absorbance of the samples was read on a microplate (ELISA) reader at 570 nm.

Flow cytometry for cell cycle and apoptosis evaluation

The logarithmic-phase cells were routinely dissociated into single cell suspensions placed in a 6-well plate (2 mL of cell suspension per well at a density of 5×10^5 cells/mL) and allowed 16 h for attachment. Then, cells were harvested after treatment with increasing concentrations (0.1, 0.5, 1, 2, or 5 µmol/L) of AL3818 for 12 h, followed by fixation in 70% cold ethanol. Finally, cells were stained using propidium iodide (PI) staining solution and incubated in dark conditions for 30 min. After staining, the PI-positive cells were detected by flow cytometry analysis (excitation set at 405 nm; emission at 450 nm) and the results were analysed by use of Cell Quest (Becton, Dickinson and Company, USA) and ModFit software (Verity Software House, USA).

Western blot analysis

Western blot was performed to detect the levels of TLR-4, NF-κB, VEGF, survivin, and Bax proteins in each studied cell group. After 48 h treatment with AL3818 (0.1, 0.5, 1, 2, or 5 µmol/L), PANC-1 cells were washed three times in pre-cooled phosphate-buffered saline (PBS) and lysed on ice for 30 min. After centrifugation (12,000x g for 10 min) at 4°C, the supernatant was taken for analysis of the protein concentrations by the bicinchoninic acid (BCA) assay (Bio-Rad Laboratories, CA, USA). A total of 50 µg of cellular proteins was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and then transferred onto poly-vinylidene difluoride (PVDF) membranes. After blocking, the membranes were immunolabeled with primary antibodies against TLR-4 (1:1000), NF-κB (1:1000), VEGF (1:1000), survivin (1:5000), and Bax (1:1000) (Abcam, UK) at 4°C overnight. The membranes were washed three times with TBST (Tris-buffered saline with 0.1% Tween 20) and then incubated with secondary antibodies – Goat Anti-Rabbit IgG (1:10000) or Rabbit Anti-Mouse IgG (1:40000) (Abcam, UK) for 2 h at room temperature. After washing three times in TBST, electrochemiluminescence was used to visualize the blots and protein signals were captured and documented on X-ray films. Beta-actin served as an internal reference marker. Target protein expression levels were determined based on the relative ratio of the grayscale values between the target protein band and the β-actin internal reference. The relative expression levels for each treatment group were calculated compared to those of the control (untreated) group (assumed to be 100%).

Statistical analysis

All data were presented as means ± standard deviation (SD). For comparisons among experimental groups, the one-way analysis of variance (ANOVA), the Least significant difference (LSD), and the Student-Newman-Keuls (SNK) tests were used, as appropriate. The significance level was set to $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

Results and Discussion

Effects of AL3818 on cell morphology

The cells in the control (untreated) group presented different shapes, either round or oval, with round enlarged nuclei and a high nuclear-cytoplasmic (N/C) ratio. By contrast, the AL3818-treated cells tended to have quite uniform sizes and shapes with smaller nuclei of higher density and a decreased N/C ratio. Compared to the control group, the treated groups

displayed lower cell proliferation and division rates with some dead (apoptotic and necrotic) cells. Cell number and growth rates were significantly reduced in the presence of AL3818 (Figure 1).

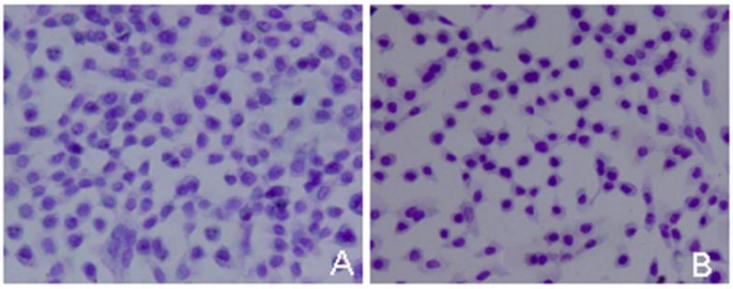

Figure 1.
Microscopy imaging of cellular morphology before (A) and after exposure to 1.0 µmol/L AL3818 (B) (HE stain, 400x)

Antiproliferative effect of AL3818 against PANC-1 cell line

An MTT assay was carried out as an indicator of cell proliferation. As shown in Table I, compared with control cells (0 µmol/L), AL3818 could significantly suppress the growth of PANC-1 cells in a dose-dependent manner.

Table I
Antiproliferative effect of AL3818 on pancreatic cancer cell line PANC-1

AL3818 dose (µmol/L)	**0**	**0.1**	**0.5**	**1.0**	**2.0**	**5.0**	***p-value***
Cell survival rate, mean ± SD	0.99 ± 0.09	0.93 ± 0.09	0.89 ± 0.07	0.72 ± 0.07	0.60 ± 0.06	0.42 ± 0.07	*< 0.001*

Evaluation of cell cycle and apoptosis by flow cytometry

To investigate the antiproliferative function of AL3818, the cell cycle distribution was evaluated. After 48 h of AL3818 treatment, PANC-1 cells were analysed by flow cytometry. As shown in Table II, compared with control cells, the proportion of G0/G1 and S phase cells significantly decreased ($p < 0.05$), while the fraction of G2/M phase cells increased ($p < 0.05$), suggesting that AL3818 induced cell cycle arrest during the G2/M phase in the treated cells. The percentage of G2/M phase and apoptotic cells increased in a dose-dependent manner.

Table II
Cell cycle analysis of PANC-1 cells after 48 h of AL3818 treatment

AL3818 dose	**G0/G1 phase,** mean ± SD	**S phase,** mean ± SD	**G2/M phase,** mean ± SD	**Apoptosis rate,** mean ± SD
0 µmol/L	38.03 ± 1.07	45.28 ± 2.28	16.69 ± 1.04	0.03 ± 0.01
0.1 µmol/L	35.17 ± 1.38	42.18 ± 2.03	22.65 ± 1.72*	3.89 ± 0.28*
0.5 µmol/L	33.87 ± 1.26*	40.07 ± 1.71*	26.06 ± 2.17*	8.27 ± 0.51*
1 µmol/L	30.14 ± 2.19*	38.98 ± 1.88*	30.88 ± 1.12*	15.33 ± 1.63**
2 µmol/L	28.84 ± 2.05*	36.14 ± 2.11*	35.02 ± 1.85**	18.64 ± 2.06**
5 µmol/L	27.06 ± 1.93*	32.81 ± 1.58*	40.16 ± 1.59**	21.59 ± 2.74**

*$p < 0.05$, **$p < 0.01$ compared to control group

Protein expression profiles by Western Blot

The TLR-4, NF-κB, VEGF, survivin and Bax protein expression levels in the cell supernatant of each group were analysed by Western Blot. As shown in Table III, the expression of TLR-4, NF-κB, VEGF and survivin was significantly downregulated in cells exposed to AL3818 ($p < 0.05$), while the expression of Bax was upregulated considerably ($p < 0.05$) in a dose-dependent manner.

Table III
Expression levels of TLR-4, NF-κB, VEGF, survivin and Bax proteins in control and AL3818-treated PANC-1 cells

AL3818 dose	**TLR-4 (ng/mL),** mean ± SD	**NF-κB,** mean ± SD	**VEGF,** mean ± SD	**Survivin,** mean ± SD	**Bax,** mean ± SD
0 µmol/L	100.00 ± 1.04	100.00 ± 1.13	100.00 ± 1.52	100.00 ± 1.64	100.00 ± 1.39
0.1 µmol/L	58.93 ± 1.31**	93.15 ± 1.42	75.42 ± 1.27*	80.38 ± 1.08*	109.16 ± 0.62
0.5 µmol/L	47.19 ± 1.04**	86.37 ± 1.82*	64.28 ± 1.16*	67.64 ± 1.43*	118.37 ± 1.48*
1 µmol/L	42.73 ± 2.05**	63.19 ± 1.49*	53.26 ± 1.04**	50.22 ± 1.16**	157.27 ± 2.93**
2 µmol/L	37.16 ± 1.27**	47.82 ± 0.93**	47.22 ± 0.94**	43.29 ± 1.39**	171.42 ± 3.67**
5 µmol/L	32.88 ± 1.08**	29.68 ± 0.74**	44.13 ± 1.02**	31.65 ± 1.02**	183.25 ± 3.59**

*$p < 0.05$, **$p < 0.01$ compared to control group

Pancreatic cancer remains one of the leading causes of global cancer-related deaths and one of the cancers with the poorest prognosis [13]. In recent years, the rapidly developing technology of molecular biology has allowed a deeper understanding of cancer biology at the cellular and molecular level, paving the way for molecularly targeted anticancer therapies. As one of the cornerstones of precision medicine, molecular-targeted therapy can interfere with specific molecules or biologic pathways involved in cancer development with minimal harm to normal cells [14, 15]. Tumour metastasis is one of the main biological characteristics of a malignant tumour, and the metastatic potential of the cancer is one of the manifestations of tumour heterogeneity. Tumour heterogeneity raises many problems for targeted therapy, and maybe the combination of targeted anticancer drugs can overcome the heterogeneity of cancer [16, 17]. Currently, it has been found that the inflammatory factor prostaglandin E-2 (PGE-2) promotes the occurrence and development of tumours by remodelling the tumour microenvironment [18]. In addition, PGE-2 also increases the resistance of tumour cells to treatment by promoting tumour proliferation and epithelial-mesenchymal transition [19]. Ketoconazole is a kind of imidazole broad-spectrum antifungal drug which has been applied in the prevention of experimental metastasis of pancreatic cancer. The results show that it may have a certain impact on the synthesis of prostaglandins [20]. In this field, AL3818, as a new type of small molecule multi-target TKI, has demonstrated promising antitumor activity in various types of advanced malignancies [21].

Our study employed the human PANC-1 cell line to preclinically explore the effects of AL3818 on the proliferation and apoptosis of pancreatic cancer cells and the potential mechanisms underlying its antitumor capacity. Based on the MTT assay results, AL3818 was found to exert significant, dose-dependent anti-proliferative effects *in vitro* ($p < 0.05$). Analysis of cell cycle changes and apoptosis rates using flow cytometry and PI staining revealed that cell cycle and apoptosis showed significant alterations in the presence of AL3818, depending on the dose and duration of treatment. It has been reported that cell cycle arrest and apoptosis are closely related [22]. Moreover, anti-neoplastic agents have been shown to regulate tumour cell proliferation by inhibiting cell cycle progression and promoting cell differentiation and apoptosis [23, 24]. Our findings indicated that AL3818 could arrest PANC-1 cells in the G2/M phase of the cell cycle, suggesting that AL3818 could suppress proliferation and/or induce apoptotic death in pancreatic cancer cell lines.

As the first reported TLR, TLR-4 is known to play a crucial role in many aspects of the innate immune responses resulting in the release of pro-inflammatory mediators [25]. The TLR-4 signalling has been increasingly recognized as a critical factor involved in tumour pathogenesis, including pancreatic malignancies, by regulating tumour cell proliferation, apoptosis, and invasion [26-28]. Indeed, overexpression of the TLR-4 pathway and its essential component NF-κB in the pancreatic tumour microenvironment has previously been reported. Of note, a significant association between elevated TLR-4 expression and poor patients' survival has also been supported [28]. This study demonstrated that AL3818 could reduce the expression of TLR-4, NF-κB, VEGF and survivin while increasing the expression of Bax in a dose-dependent manner. The VEGF family can stimulate angiogenesis in neoplastic tissues, allowing tumour cell proliferation and metastatic spread [29, 30]. Therefore, our data further supported the apoptosis-inducing effects of AL3818, which may be a key strategy for preventing tumour recurrence and metastasis.

Conclusions

The reported findings suggested that AL3818 can exert significant antiproliferative and apoptotic effects on the PANC-1 cell line *in vitro*. Moreover, cell cycle arrest at the G2/M phase following treatment with AL3818 may result from alterations in the expression and activity of the TLR4/NF-κB signalling pathway, VEGF, surviving and/or Bax. Our study has thus provided evidence for the initial preclinical evaluation, as well as the potential utilization of AL3818 in the setting of pancreatic cancer. Further research is clearly needed to validate these results and expand our understanding of *in vitro* models for translational research and drug discovery in precision oncology for pancreatic carcinogenesis.

Conflict of interest

The authors declare no conflict of interest.

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