FNC Inhibits Proliferation and Metastasis of Non-Small-Cell Lung Cancer in Vivo and In Vitro

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Research

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Abstract

Background: Non-small-cell lung cancer (NSCLC) is associated with the highest morbidity and mortality rate, and chemotherapy is the primary therapy for NSCLC. However, the clinical outcome is still far from satisfactory. This study aimed to determine the effects and mechanisms of FNC on NSCLC.

Results: FNC, 2'-deoxy-2'- β -fluoro-4'-azidocytidine, is a novel cytidine analogue, inhibits the proliferation and metastasis of H460 cells in a time- and dose-dependent manner in vitro. Mechanistically, treatment of H460 cells with FNC inhibits Bcl-2 expression and potentiates Cytochrome C (Cyt-C) release, Bax and caspase-3 expression. In parallel, FNC inhibits tumor migration, invasion and metastasis in H460 cells by increasing E-cadherin protein levels and reducing the expression of VEGF, MMP-2, MMP-9 proteins. And murine models and mouse xenograft models also proved that FNC could significantly inhibit NSCLC tumor growth and metastasis with low toxicity. Finally, we also discovered that inhibition of DNMT3B expression is one of the important mechanisms of FNC inhibiting proliferation and metastasis of NSCLC cells.

Conclusions: FNC induces apoptosis through the mitochondrial apoptotic pathway and represses tumor progression by targeting multiple proteins related to cell adhesion and invasion in vitro and in vivo. Meanwhile, FNC was involved in regulating DNA methylation. In summary, our results support the further development of FNC as potential therapeutics for clinical non-small cell lung cancer.

Introduction

Lung cancer is a frequently diagnosed malignancy with the highest morbidity and mortality around the world [1]. The incidence of lung cancer is increasing year by year, the prognosis is poor, and the 5-year survival rate is less than 15%. NSCLC accounts for approximately 85% of all lung cancer cases [2]. Surgical resection is the most effective treatment for specific cases of NSCLC in stages I to II and IIIA [3]. For patients with advanced NSCLC, platinum-dual-line chemotherapy (PT-DC) can improve prognosis, with an objective response rate (ORR) of about 26%, while the 5-year survival rate is only about 5% [4]. After simultaneous treatment with epidermal growth factor tyrosine kinase inhibitors, almost all patients eventually developed the disease due to acquired drug resistance [5]. Therefore, development of new therapeutic drugs is urgently needed for the treatment of NSCLC.

Nucleoside analogs belong to a group of important antitumor drugs [6]. Nucleoside analogs exert their cytotoxic effects by mimicking endogenous nucleosides [7]. FNC is a novel small molecule nucleoside analog with independent research and development patents [8]. Previous studies have shown that FNC exhibits antitumor activity in many types of lymphomas [9, 10]. But for the treatment of FNC on NSCLC, there is no clear conclusion on how does FNC generate the antitumor effects. Therefore, we conducted related experiments to investigate whether the antitumor effect has a relationship with apoptosis and metastasis after FNC treatment. In this study, we aimed to confirm the effects of FNC on NSCLC and the

potential mechanisms of these effects. We hope that FNC can be clinically utilized to treat NSCLC in future.

Materials And Methods

Cells and cells culture

Lewis and human H460 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Solarbio, Beijing, China) supplemented with 10% fetal bovine serum (FBS) (Gemini, USA) and 1% streptomycin/penicillin. The cells were placed in an incubator with a humidified atmosphere at 37 °C with 5% carbon dioxide.

Reagents and antibodies

The FNC was provided by Professor Junbiao Chang (Zhengzhou University, Henan, China), and the cisplatin (DDP) and gemcitabine (GEN) for injection were commercially available (Qilu Pharmaceutical co., Ltd, China). Antibodies for GAPDH, Caspase-3, Cyt-C, Bcl-2, Bax, E-cadherin, VEGF, MMP-2, MMP-9 and HRP-labeled Goat Anti-Rabbit IgG (H+L) were purchased from Proteintech Group (Wuhan, China).

MTT

H460 cells were seeded in a 96-well plate at 5000 cells per well. After the cells adhered to the culture plate and grew to 60% confluence, the medium was changed to medium that contained various concentrations of FNC. After culturing for different times, the medium was replaced and added the MTT reagent (Solarbio, Beijing, China), and cells were cultured in the incubator for 4 h. After the supernatant fluid was removed, 150 µL of solubilization solution (DMSO) was added to each well and shaken for 10 minutes. Finally, the absorbance of each well was measured by using a microplate reader. The cell viability was calculated according to the instructions from manufacturer.

Wound scratch

80–90% confluent 1×10^5 H460 cells were incubated with 0% serum medium and were scratched with a 100 µl pipette tip. RPMI 1640 medium supplemented with 10% FBS was added with FNC. The area of migration was then assessed via ImageJ (NIH, Bethesda, USA). Percentage of wound-healing was calculated as the following formula: $(\text{original scratch width} - \text{scratch width after healing}) \times (\text{original scratch width})^{-1} \times 100\%$.

Transwell

Cell migration and invasion were measured using 8-µmpore transwell compartments (Corning, USA). Fibronectin coated the back of the chamber membrane and Matrigel (BD Biosciences, USA) was added to each well before H460 cells (1×10^5) were suspended in the upper compartment. After cells were incubated at 37 °C for 24 h, the cells were incubated with 0.1% crystal violet for 30 min at room

temperature. Five images per chamber were taken using an inverted microscope and counted using ImageJ. The invasion inhibition rate was calculated as follows $\text{Invasion inhibition rate} = (1 - \text{experimental group} / \text{control group}) \times 100\%$.

Apoptosis

The H460 cells were cultured in a six-well plate at an appropriate concentration. The cells were treated with FNC for 48h after the cells grew to an appropriate density. According to the actual reagent instructions, the fibroblasts were collected, washed twice with PBS and then stained with an Annexin V-FITC kit (Keygen, Nanjing, China). All the samples were analysed with a CytExpert flow cytometer.

Animal experiments

BALB/c nude male mice and C57BL/6 male mice (5-6 weeks old; weighing 18-20 g) (Vital River Laboratory, Beijing, China), were maintained in a specific pathogen-free (SPF) environment with a 12 h light/dark cycle at 20-25°C with a relative humidity of 40–70% and received sterilized food and water freely available. Animal experiments were approved by the Animal Experimentation Ethics Committee of Zhengzhou University (Henan, China).

The right armpit of C57BL/6 male mice was subcutaneously injected with 0.067g Lewis tumor tissue suspension in 0.2ml PBS. The next day, the mice were randomized into five groups (n =10). The mice were treated with FNC (2mg / kg, 4mg / kg, 8mg / kg), DDP (2 mg / kg) or vehicle (0.9% saline) by intraperitoneal injection for 12 days (one time per day). Twelve days after treatment, the mice were euthanized with CO₂ gas. The tumors were harvested, weighed and frozen in liquid nitrogen immediately.

The back of BALB/c nude male mice was subcutaneously injected with 5×10^6 H460 cells in 0.2ml PBS. When tumor volume reached about 100 mm³, the mice were randomized into five groups (n =6). The mice were treated with FNC (2mg / kg, 4mg / kg, 8mg / kg), GEN (30 mg / kg, once every three days.) or vehicle (0.9% saline) by intraperitoneal injection for 11 days (one time per day except GEN). The tumor sizes were measured by a caliper every other day and calculated as $\text{volume} = (\text{tumor length}) \times (\text{tumor width})^2 / 2$. At the end of the experiment, mice were sacrificed by CO₂ euthanasia and the tumors were harvested, weighed, and post-fixed in 4% formaldehyde.

Statistical analysis

All data were processed by GraphPad Prism 8 Software. All values are expressed as means $\pm$ SD. One-way ANOVA was carried out to calculate the significance p-value. Values with P less than 0.05 were considered as statistical significance.

Results

FNC inhibits the growth of H460 cells in a dose- and time-dependent manner

The effect of FNC in H460 cells was measured by the MTT assay in five treated or untreated groups (0.019, 0.078, 0.313, 1.250, 5.000 μ M and control) for three different treatment times (24 h, 48 h, and 72 h), and DDP was used as a positive control drug. Here, we showed that FNC significantly inhibited the growth of H460 cells in a dose- and time-dependent manner ($p < 0.05$) (Fig. 1a). The IC₅₀ values of FNC treatment were 6.971, 0.632, and 0.267 μ M at 24 h, 48 h, and 72 h, respectively. H460 cells become more sensitive to FNC when compared to DDP (Fig. 1a).

FNC induces the apoptosis of H460 cells

To further investigate whether the growth inhibition was due to apoptosis, H460 cells were incubated with FNC (0, 0.313, 1.250, and 5.000 μ M) for 48 h and then were analyzed by flow cytometry. As shown in Fig. 1b, the percentages of apoptotic cells were significantly increased by FNC treatment in a dose-dependent manner ($p < 0.05$). The percentage of cells undergoing apoptosis was determined by the sum of the cells in early and late apoptosis.

FNC inhibits the invasion and migration of H460 cells

The effects of FNC on the motility of H460 cells were evaluated using scratch wound assays. The results of the scratch assay showed that FNC led to decreased cell migration in a dose- and time-dependent manner ($p < 0.05$) (Fig. 1c). To confirm results from wound healing experiment, we further performed Transwell assay. Transwell migration experiments displayed that FNC suppresses H460 cells migration ($p < 0.05$) (Fig. 1d). These results suggest that FNC suppresses NSCLC cell migration and invasion.

FNC effectively suppresses tumor growth in vivo

To determine the effects of FNC on lung cancer in vivo, we introduced the Lewis lung carcinoma mouse model in C57BL/6 mice. The results showed that FNC significantly inhibited tumor growth ($p < 0.05$) (Fig. 2a). FNC did not reduce mouse body weights, suggesting minimal toxicity. But the treatment of tumor-bearing mice with DDP caused a significant reduction in body weight ($p < 0.05$) (Fig. 2a). These results suggest that FNC shows more pronounced anticancer effects with little toxicity in a dose-dependent manner compared with DDP in Lewis lung carcinoma mouse model.

To further evaluate the antitumor potential of FNC in vivo, we generated a NSCLC xenograft model by subcutaneous injection of H460 cells into BALB/c nude mice. Suppression of FNC significantly attenuated tumor growth in a dose-dependent manner compared to controls ($p < 0.05$) (Fig. 2b, c). Mice weights within each treatment group showed minor fluctuations but the mice weights of treated with FNC (2mg/kg, 4mg/kg) remained within a tolerable weight loss of $< 15\%$ (Fig. 2b). These results suggest that FNC shows more pronounced anticancer effects with little toxicity in a dose-dependent manner compared with GEN in NSCLC xenograft model.

FNC induces apoptosis through the mitochondrial apoptotic pathway in vitro and in vivo

We detected the effects of FNC on the mitochondrial apoptotic pathway by WB.

In H460 cells, WB results showed that FNC induce the expression of Bax and Caspase-3 proteins and the release of Cyt-C in a dose-dependent manner ($p < 0.05$). Meanwhile, FNC inhibit the expression of Bcl-2 protein ($p < 0.05$) (Fig. 3a). In Lewis lung carcinoma mouse model, WB results showed similar results (Fig. 3b). These results indicate that FNC induces apoptosis by activating a mitochondrial apoptotic pathway in vivo and in vitro.

FNC represses tumor progression by targeting multiple proteins related to cell adhesion and invasion

EMT- and metastasis -related proteins play an important role in promoting invasion. We detected the effects of FNC on the EMT- and metastasis -related proteins by WB and IHC. In H460 cells, WB results showed that FNC induce the expression of E-cadherin protein and inhibit the expression of VEGF, MMP-2 and MMP-9 proteins in a dose-dependent manner ($p < 0.05$) (Fig. 4a). In NSCLC xenograft model, IHC results showed that FNC inhibit the expression of CD31($p < 0.05$) (Fig. 4b). These findings suggest that FNC suppresses tumor growth and progression by targeting multiple proteins related to cell adhesion and invasion in NSCLC.

FNC regulates DNA methylation in H460 cells

To study the epigenetic impact of FNC on H460 cells, we initially investigated the expression of DNMT and RAR- β genes. Semi-quantitative PCR analysis shows that FNC inhibits the expression of DNMT3B and induces the expression of RAR β ($p < 0.05$) (Fig. 4c). The results suggested that FNC altered DNA methylation patterns in NSCLC.

Discussion

Lung cancer is one of the most deadly malignant tumors in the world [11]. About 85% of lung cancer cases are diagnosed as non-small cell lung cancer [12]. For inoperable NSCLC patients, the combination of radiotherapy and chemotherapy is the main treatment [13]. However, due to factors such as chemotherapy tolerance, the prognosis of non-small cell lung cancer is still not ideal [14]. This study confirmed that FNC can effectively inhibit the process of NSCLC with low toxicity

The mitochondrial pathway is the one of the ways to induce apoptosis [15]. In the mitochondrial pathway of apoptosis, decrease in mitochondrial membrane potential promotes the release of Cyt-C from the mitochondria into the cytoplasm and activated caspase-3, caspase-7, and caspase-9 [16]. Caspases activity can effectively kill cells within minutes [17], leading to apoptosis. Bcl-2 family members, including antiapoptotic Bcl-2 and proapoptotic Bax, are critical mediators of mitochondrion-dependent apoptosis [18]. This study indicated that FNC induces apoptosis by activating mitochondrial pathway in NSCLC.

The loss of E-cadherin, a critical component for cell adhesion, is a key event in dissolution of cell-cell contacts during EMT [19]. Matrix metalloproteinases (MMP) comprise a super-family of zinc-dependent endopeptidases, and the expression of MMP-2 and MMP-9, associated with tumor progression and metastasis, was also increased [20]. VEGF is related to angiogenesis, and tumor cells secrete VEGF to

ensure adequate blood supply. Over-expression of VEGF can facilitate tumor growth due to neovascularization [21]. CD31 expression was significantly correlated with majority of vascular endothelial cell markers. It has been well documented that CD31 which is widely used to assess angiogenesis, the highly expressed CD31 indicates active proliferation of endothelial cells [22]. This study demonstrated that FNC could inhibit the metastasis in NSCLC by regulating the expressions of multiple proteins related to cell adhesion and invasion.

It is well recognized that DNA methylation is mediated by DNA methyltransferases, such as DNMT3A and DNMT3B, which are de novo DNA methyltransferases [23]. Aberrant DNA methylation may result in both upregulation of oncogenes and the silencing of tumor suppressor genes [24]. RAR β is a potential tumor suppressor gene, expressed in the lung, bronchus, and reproductive system, and is related to tumor differentiation and malignancy [25]. The hypermethylation of the RAR β promoter in NSCLC is significantly increased, which indicates that RAR β methylation contributes to the development of NSCLC [26-28]. In this study, we discovered that FNC inhibits the expression of DNMT3B gene and promotes the expression of RAR β gene in H460 cells. In future studies, methylation status of more genes should be further investigated to identify the relationships between their methylation status and FNC.

Conclusion

In summary, our study shows that FNC can inhibit NSCLC cells proliferation, and this effect is related to activation of NSCLC cells apoptosis. In addition, FNC regulated the related protein expressions of adhesion and invasion, which may be the potential mechanism by which FNC treatment on NSCLC. As a cytidine analogue, FNC can induce NSCLC cells apoptosis and inhibit tumor metastasis in vitro and in vivo, which might be a new approach for treating NSCLC.

Abbreviations

FNC: 2'-deoxy-2'- β -fluoro-4'-azidocytidine; NSCLC: Non-small-cell lung cancer; ORR: Objective response rate; Cyt-C: Cytochrome C; VEGF: Vascular endothelial growth factor; MMP2: Matrix Metalloproteinase 2; MMP9: Matrix Metalloproteinase 9; DNMT3B: DNA methyltransferase 3B; DNMT1: DNA methyltransferase 1; DNMT3A: DNA methyltransferase 3A;

Declarations

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Authorship contribution statement

XJ and SN performed the in vivo and in vitro experiments and drafted the

manuscript. YL participated in the in vivo experiments and the reversion of this manuscript. HC, NW, YP, FM and WY participated in the in vitro studies and performed the statistical analysis. QW and JC participated in the design of this study. YZ and YZ conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

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Availability of supporting data

All data and materials are available upon request.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Experimentation Ethics Committee of Zhengzhou University (Henan, China).

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare no competing financial interests

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Supplementary Information

Supplementary Material.docx

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