Neferine, A Novel ROCK1 Targeting Inhibitor, Blocks EMT Process and Induces Apoptosis in Non-Small Cell Lung Cancer

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Abstract

The compounds derived from Traditional Chinese Medicines have shown various pharmacological activities with unique advantages, especially in the aspect of anti-tumor. Neferine (Nef), a natural compound, extracted from green seed embryos of Lotus (*Nelumbo nucifera* Gaertn) also exerts antitumor effects on cancers. In this study, the effects and mechanisms of Nef on epithelial-to-mesenchymal transition (EMT) process in non-small cell lung cancer (NSCLC) were evaluated. The results showed that Nef had the antitumor effects *in vivo* and *in vitro*. Nef significantly suppressed cell viability and induced apoptosis in NSCLC cells, with elevated reactive oxygen species and reduced BCL-2 /BAX ratio. Nef was also demonstrated to inhibit the invasion, metastasis and EMT process of NSCLC cells, and attenuate EMT-related changes of E-cadherin, N-cadherin and Vimentin at both transcriptional and translational levels. Moreover, we concluded that the inhibitory effects of Nef on EMT was achieved by targeting Rho-associated protein kinase 1 (ROCK1), a protein mediating the process of EMT in various cancers. These results showed that Nef had a significant antitumor effect on NSCLC cells by inducing apoptosis and blocking EMT, providing the therapeutical prospect on NSCLC treatment.

Introduction

Lung cancer is the most frequent cause of cancer deaths worldwide and the non-small cell lung cancer (NSCLC) constitutes approximately 85% of lung cancers. The treatment of NSCLC has made a significant progress, with the advent and application of small molecule tyrosine kinase inhibitors and immunotherapy over the past two decades (Herbst et al. 2018). However, the characteristics of metastasis and epithelial-to-mesenchymal transition (EMT) process are still the barrier of NSCLC cure (Herbst et al. 2018; Ou and Zhu 2019). These traits increase the resistance and recurrence of NSCLC (Zheng et al. 2018).

Increasing evidence demonstrates that onco-factor KRAS promotes the metastasis and EMT process in NSCLC cells (Adachi et al. 2020; Larsen et al. 2016; Shaurova et al. 2020). KRAS mutations are the most frequent gain-of-function alterations and at least 1/4 of NSCLC patients have KRAS mutation (Adderley et al. 2019). Metastasis and recurrence are common in KRAS-mutated lung cancer (Renaud et al. 2015; Renaud et al. 2016). A study on metastasis of KRAS mutant lung cancer cells showed that wild-type KRAS was detected in primary tumor sites and then detectable mutant KRAS was present in metastatic sites, supporting the important role of KRAS mutations in metastasis of cancer cells (Rau et al. 2016). In addition, after the occurrence of KRAS mutation and metastasis, the response of tumor cells to treatment of tyrosine kinase inhibitors is reduced, which also indicates that KRAS-induced metastasis is extremely threatening to the survival of lung cancer patients (Rau et al. 2016). Previous studies highlight the inhibition of Rho-associated protein kinase (ROCK), the downstream of GATA2, which has a synthetic lethal interaction between oncogenic KRAS can result in dramatic tumor inhibition, especially in combination with bortezomib (Barbacid 2012; Kumar et al. 2012). ROCK is a serine/threonine kinase, involved in cytoskeleton organization, cell adhesion and motility, and can promote metastasis of tumor by phosphorylation of myosin light chain (Hartmann et al. 2015). Overexpression of aberrant-activation

of ROCK is associated with the poor diagnosis and shortened survival of cancer patients (Deng et al. 2017). The modulation of ROCK in EMT in hepatocellular carcinoma, osteosarcoma and NSCLC has been suggested as the potential therapy target of cancers (Guo et al. 2019; Lin et al. 2018; Shi et al. 2019). However, there is less study on the effects of ROCK in metastasis of KRAS mutant NSCLC although it is considered as a therapeutical target.

Nowadays, the natural compounds derived from Traditional Chinese Medicines (TCMs) have shown the potential treatment value on tumors (Zhang et al. 2020). Neferine (Nef), a natural compound, extracted from green seed embryos of Lotus (*Nelumbo nucifera* Gaertn) also exerts various function on various tumor cells, such as proliferation inhibition, apoptosis induction, and mitochondrial dysfunction (Erdogan and Turkekul 2020; Wang et al. 2020; Xie et al. 2020). It also indicates the inhibitory effects of Nef on lung cancer, including suppression on lung carcinogenesis and sensitization of chemotherapy (Sivalingam et al. 2019; Sivalingam et al. 2017). However, the effects and mechanism of Nef on EMT process in NSCLC still unknown. Herein, the KRAS mutant NSCLC cells A549 was used to determine the *in vitro* cytotoxicity and inhibitory ability of Nef on EMT in comparison with H1299 cells (NRAS mutant NSCLC), and found that Nef had ROCK1 targeting affinity via molecular docking and as well as the role of ROCK1 in metastasis of NSCLC.

Materials And Methods

Cell culture and reagents

The NSCLC cells lines (A549, and H1299) and human normal lung epithelial cells BEAS-2B purchased from KeyGen BioTECH (Nanjing, China) were incubated in RPMI-1640 medium (KeyGEN BioTECH, NanJing, China) contains 10% fetal bovine serum (FBS; WISENT corporation, Wisent, Canada), as well as 1% penicillin (WISENT) and 1% streptomycin (WISENT). The cells were incubated in Heracell 150i Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO_2 atmosphere. Trypin was purchased from WISENT. Neferine (Nef, $C_{38}H_{44}N_2O_6$, Molecular weight: 624.8, Purity> 95%), obtained from Nanjing University of Chinese Medicine (Nanjing, China), and the molecular structure were shown in **Figure 1A**. Nef were dissolved in dimethyl sulfoxide (DMSO) to produce 10 mM stock solution and stored at -80°C. The primary antibodies including B-cell lymphoma-2 (BCL2) (#48496), BCL2 associated X protein (BAX) (#29057), ROCK1 (#48890), GAPDH (#21612), E-cadherin (#40860), N-cadherin (#48495), Vimentin (#41531) were purchased from Signalway Antibody (MD, USA), and the HRP-conjugated Goat anti-Rabbit (#S0001) were purchased from Affinity Biosciences (Cincinnati, OH, USA). All antibodies were used in accordance with the instructions. Recombinant human transforming growth factor-β1 (TGF-β1) was purchased from STEMCELL Technologies (Vancouver, BC, Canada).

In vivo assay

The *BALB/c* mice (male, 6 weeks old, weighing 18~22 g) (Gempharmatech, Jiangsu, China) were engrafted in the right legs of the mice with A549 cellsor TGF-β1-treated A549 cells (5×10^6 cells) in PBS.

The mice were divided into six groups as shown in Figures. The mice were received Nef intraperitoneally for everyday for three weeks. The tumor volumn ($V=1/2\times L\times D^2$, mm^3) and mice weight (g) were measured. Animal experiments were approved by the Review Board of Nanjing University of Chinese Medicine (Ethics number: 202011A002).

Cell transfection

Scrambled siRNA and *ROCK1* siRNA were designed and synthesized by GenePharma (Shanghai, China). For cell transfection, the cells were transfected with 2.5 μg plasmid or 50 nM siRNA using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. After transfection for 48 h, the cells were collected and used for the subsequent experiments.The primers of *ROCK1* siRNA were as follows: F:CCAGUUGUACCCGAUUUAATT; R:UUAAAUCGGGUACAACUGGTT

Cell apoptosis

The cells were collected and labeled with Annexin V and PI according to the protocols of Annexin V/PI Cell Apoptosis Detection Kit (Vazyme biotec, Nanjing, China). The fluorescence was detected by BectoneDickinson FACS Calibur flow cytometry (NJ, USA) and the data analysis was performed by Flowjo software (Tree Star, Ashland, OR, USA).

Reactive oxygen species (ROS) detection

The cells after drug treatment were harvested and stained with DCFH-DA (10 mM, diluted in serum-free medium; Beyotime Biotechnology, Shanghai, China) for 20 min at 37°C. Then cells were washed with phosphate-buffered saline (PBS) for 3 times. Finally, the cells were resuspended in PBS and detected by fluorescence microscope (Lecia, Wetzlar, Germany).

Cell viability

The cell viability was determined by CCK8 Cell Counting Kit (Vazyme biotec). The cells growth in 96-well plate were treated for 0, 4, 6, 10, 20, 40 and 80 μM of Nef for 24 or 48 h and then incubated with 100 μL of CCK8 solution for 30 min at 37°C in a 5% CO_2 atmosphere. The optical density (OD) was detected by VICTOR Multilabel Plate Reader (PerkinElmer, Hopkinton, USA). The cell viability was calculated as follows: Cell viability (%) = $(OD_{test}-OD_{blank})/(OD_{control}-OD_{blank})\times 100\%$.

Western blot

The total protein was extracted from the cells treated with Nef using RIPA Buffer (Beyotime Biotechnology) and the concentration of protein were determined by BCA Protein Assay Reagent (Thermo Fisher Scientific). Protein samples (40 μg each) were loaded, separated by SDS-PAGE gels and transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, USA) followed by western blot analysis. Immunodetection was performed using a WesternBright ECL (Advansta, Bering Drive San Jose, CA, USA). Detection was performed with ChemiDoc XRS+ System (Bio-Rad) and captured by Image Lab software

(Bio-Rad). The bands were quantified by Image J software (National Institutes of Health, Bethesda, MD, USA). The blots were representative of triple independent experiments.

Immunofluorescent assay

The cells after treatment were washed by PBS and then fixed by 4% paraformaldehyde for 30 min. The cells were permeabilized in 0.3% Triton X-100 for 30 min. After blocking with 3% BSA and incubating with primary antibodies, the cells were followed by incubation with a secondary antibody conjugated to Alexa Fluor (Bio-Rad) for 1 h and DAPI (Bio-Rad). The cell images were captured with a confocal laser scanning microscope (Leica).

Real-time quantitative polymerase chain reaction (qRT-qPCR) analysis

Total RNA was extracted from tissue samples and cells using RNA Easy Fast Tissue/Cell Kit (TIANGEN BIOTECH, Beijing, China) according to manufacturer's instructions. RNA was reversely-transcribed into cDNA using the FastKing gDNA Dispelling RT SuperMix Kit (TIANGEN BIOTECH). qPCR was performed using iTaq™ Universal SYBR® Green (Bio-chain, Shanghai, China) and CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The thermocycling conditions were as follows: 40 cycles at 95°C for 30 sec (denaturation), 60°C for 10 s (annealing), and 72°C for 30 s (extension). GAPDH was served as an internal control. Relative gene expression was quantified using $2^{-\Delta\Delta Ct}$ method. The primers were as follows:

GAPDH (5'-3') F: GGAGCGAGATCCCTCCAAAAT; R: GGCTGTTGTCATACTTCTCATGG

ROCK1 (5'-3') F: AACATGCTGCTGGATAAATCTGG; R: TGTATCACATCGTACCATGCCT

E-cadherin (5'-3') F: ATTTTCCCTCGACACCCGAT; R: TCCCAGGCGTAGACCAAGA

N-cadherin (5'-3') F: TCAGGCTGTGGACATAGAAACC; R: GCTGTAAACGACTCTGGCACT

Vimentin (5'-3') F: AGTCCACTGAGTACCGGAGAC; R: CATTTCACGCATCTGGCGTTC

Transwell assay

For cell migration assay, after Nef treatment, the 200 μL cells were suspended by serum-free medium in 1×10^6/mL and loaded into the upper chamber of transwell, while 600 μL supplemented with 10% FBS was added to the lower chamber. Following incubation for 24 h, the cells in the upper chamber were removed by a cotton swab and the cells in the lower chamber were fixed by 4% paraformaldehyde and then stained with 0.1% crystal violet for 15 min at room temperature. For cell invasion assay, transwell chambers with 8-μm pore sizes (Millipore-Sigma, Boston, MA, USA) were coated with 50 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and incubated at 37°C. Finally, the cells were counted and photographed under an inverted microscope (Leica). The migration and invasion ratio were calculated by Image J software.

Wound healing test

The cells were plated in 6-well plate and cultured until 90% confluence. A 10 μL pipette tip was utilized to scratch a line wound. The cells were washed by PBS for 2 times softly. The cells were treated with 0, 4, 6 and 10 μM Nef with serum-free medium for 24 h. The line wound was imaged at 0 and 24 h of culture under an inverted microscope. The line wound area was calculated by Image J software and as follows: Wound healing (%) = ($Area_{0\ h}$-$Area_{24\ h}$)/ $Area_{0\ h}$×100%.

Enzyme linked immunosorbent assay (ELISA)

Concentrations of matrix metalloproteinase (MMP) 2 and MMP9 in the media of cells with Nef treatment were determined by Human MMP-2/9 ELISA (Jin Yibai Biological Technology, Nanjing, China) according to the manufacturer's instructions.

Molecular docking

The structural 3D model of ROCK1 – Y27632 complex (Protein Data Bank ID, 2ETR) was used to determine the interactions of ROCK1 and Nef, and the results were analyzed using AutoDock Vina software (the Scripps Research Institute, USA). The structures of Y27632 and Nef were established by Chemdraw software (Perkinelmer, Hopkinton, USA). Grid Box (40 Å ×40 Å ×40 Å) centered at (51.530, 108.818, 28.061) Å, for AcROCK1 – Y27632; grid box (40 Å ×40 Å ×40 Å) centered at (-0.088, 127.637, 22.682) Å, for Bc-ROCK – Y27632. In this condition, the conformation of the complex formed by the docking of Y27632 drawn by Chemdraw and ROCK1 was consistent with that of the original ligand complex (**Supplementary Figure 1A-D**), indicating it was suitably used in the docking experiments by utilizing the AutoDock tools.

Statistical analysis

The data performed with three independent experiments and were analyzed by GraphPad Prism 9 software (San Diego, CA, USA). For comparisons of two groups, a two-tailed unpaired *t*-test was used. For comparisons of multiple groups, one-way analysis of variance (ANOVA) was used. All data were shown as mean ± SD, and $P< 0.05$ was considered to indicate a statistically significant difference.

Results

The cytotoxicity effect of Nef in NSCLC cells.

In order to examine the antitumor effect of Nef, the cytotoxicity effect of Nef was firstly evaluated in NSCLC cells, *i.e.* A549 and H1299 cells after 24 or 48 h of treatment in a concentration range of 0-80 μM of Nef, using CCK8 assay. As shown in **Figure 1B**, the A549 and H1299 cells displayed a significantly decreased cell viability in a time- and concentration-dependent manner in response to Nef treatment. The half maximal inhibitory concentrations (IC_{50}) of Nef to A549 and H1299 were 41.03 μM and 63.30 μM at 24 h, and 14.34 μM and 26.99 μM at 48 h, respectively (**Figure 1C**). However, the normal lung cells (BEAS-2B) were less effected by Nef. Although the cell viability was decreased in high concentration of Nef for

24 h, the cell viability was recovered at 48 h (**Figure 1D**). The results showed that Nef had a cytotoxicity effect on NSCLC cells, which could be tumor-dependent, and A549 cells were slightly more sensitive to the H1299 cells. The data also suggest that Nef molecule has a relatively long half-life, as one-off addition of the Nef resulted in much higher proportions of cell death after 48 h than 24 h. In addition, we also determined the antitumor effects of Nef *in vivo*. As shown in **Figure 1E**, TGF-β1 could enhance the proliferation of xenograft tumor, while Nef could decreased the tumor growth compared with control group or TGF-β1-induced group. Besides, the antitumor effects of Nef were more significant than Fas with the less effects on mice weight (**Figure 1F**).

Nef promoted the cell apoptosis in NSCLC cells.

Previous studies indicated that Nef was able to induce cell apoptosis in prostate cancer cells and renal cancer cells (Kim et al. 2019; Nazim et al. 2020). Therefore, we also determined the potential of Nef on inducing apoptosis in NSCLC cells using Annexin V/PI Cell Apoptosis Detection Kit. After treatment with 0, 10, 20 and 40 μM of Nef for 48 h, the total apoptosis rates (Annexin V-positive cells) in A549 cells were 8.82 ± 0.36 %, 14.05 ± 0.59 %, 19.21 ± 0.51 %, and 26.25 ± 2.25%, and in H1299 cells were 11.15 ± 0.76 %, 14.51 ± 0.92 %, 23.38 ± 1.45 %, and 26.22 ± 1.34 % (**Figure 2A**). These data demonstrate that both A549 and H1299 NSCLC cells displayed a comparable apoptotic profile in response to Nef treatment.

As excess cellular levels of ROS could occur during activation of cell death, in addition, we detected that Nef could increase the ROS levels both in A549 and H1299 cells, suggesting Nef promoted the oxidative stress in NSCLC cells (**Figure 2B**). Interestingly, significantly higher levels of ROS were detected in A549 cells than in H2199 cells (**Figure 2B**) under10 or 20 μM of Nef treatment. Compelling evidence in the literature suggests that apoptosis is tightly regulated by the balance of a negative regulator, BCL2, and a positive regulator, BAX (Zhang et al. 2018). The BCL2 and BAX protein contents were subsequently analyzed by immunoblotting. The A549 and H1299 cells treated with Nef had an increased expression of BAX protein, but decreased expression of BCL2 (**Figure 2C-D**), leading to lower ratios of BCL2/BAX. However, the Nef-mediated upregulation of the BAX in response to 20 or 40 μM of treatment was more dramatic in A549 cells than in H1299 cells, indicating that there could be subtle differences in mechanisms of Nef-mediating apoptotic effects between the two NSCLC cell lines.

The inhibitory effects of Nef on cell invasion and migration of NSCLC cells.

The metastasis of cancer cells is a risk factor for tumor-associated lethality and poor prognosis in NSCLC (Huang et al. 2019). It is crucial to reduce/block the metastasis of NSCLC cells in clinical treatment and we therefore next addressed whether Nef could inhibit the invasion and migration of tumor cells *in vitro*. As shown in **Figure 3A-B**, 4, 6 and 10 μM of Nef were able to suppress the migration ability of A549 and H1299 cells in the wound healing assay (**Figure 3A**), and invasion ability with transwell assay(**Figure 3B**) in a dosage-dependent manner. We then determined the primary mechanism of metastasis inhibition of Nef. MMP2 and MMP9 are widely found to corelate with the pathology of cancers, including but not limited to invasion and metastasis (Huang 2018; Scheau et al. 2019). Nef at 4, 6 and 10 μM were found to suppress the secretion of MMP2 and MMP9 (**Figure 3C**). These results proved

the inhibitory effects of Nef on invasion and migration of NSCLC cells, which might be partially achieved by inhibiting MMP2 and MMP9.

Nef suppressed the EMT process in NSCLC cells.

The transdifferentiation of epithelial cells into motile mesenchymal cells, a process known as EMT, which plays an important role in cell progression and metastasis (Du and Shim 2016; Lamouille et al. 2014). An EMT model can be established by classic inducer TGF-β1 (Mo et al. 2017). Here we established the NSCLC EMT model *in vitro* using A549 and H1299 cells. As shown in **Figure 4A** the morphology of H1299 cells was changed after the stimulation of 5, 10 and 15 ng/mL of TGF-β1 for 12 and 24 h. The formation of invasive pseudopodia in tumor cells could be induced (arrows in **Figure 4A**; the data of A549 cells were not shown). In addition, TGF-β1 modulated the expression of EMT-related protein, including increased expression of N-cadherin and Vimentin, as well as the decreased expression of E-cadherin (**Figure 4B**). It suggested TGF-β1 promoted EMT progression in NSCLC cells. After the NSCLC EMT model establishment, we explored the effects of Nef on EMT process. As shown in **Figure 4B**, in comparison to the EMT model group treated with TGF-β1 alone, Nef could counteract the effect of TGF-β1 on expression of EMT-related proteins, *i.e.*, increased E- cadherin expression and reduced expression of N-cadherin and Vimentin at both transcriptional (**Figure 4C**) and translational levels (**Figure 4B**). This evidence suggest that Nef can inhibit EMT process in cellular and molecular levels in NSCLC cells by regulating EMT-related factors.

Nef inhibited invasion, migration and EMT process of NSCLC cells via targeting ROCK1.

Previous studies have emphasized the vital role of ROCK1 in metastasis and EMT in cancer cells (Duan et al. 2020; Li et al. 2020; Tang et al. 2019). We also found the modulation effects of ROCK1 in NSCLC cells. First, after treatment with *ROCK1* siRNA (**Figure 5A-B**), the invasion and migration ability of both A549 and H1299 cells were decreased (**Figure 5C-D**), suggesting the effects of ROCK1 in promoting metastasis in NSCLC cells. Second, ROCK1 protein expression in A549 and H1299 cells treated with EMT inducer TGF-β1 were enhanced significantly (**Figure 6A**). However, after treatment with 4, 6 and 10 μM of Nef, the ROCK1 protein expression was attenuated (**Figure 6B**), which was also proved by immunofluorescent assay (**Figure 6C**). We also determined the expression of ROCK1 in xenograft tumor in **Figure 1**. The results showed that ROCK1 expression was induced by TGF-β1 and was decreased by Nef (**Figure 6D**). These results demonstrated the correlation of ROCK1 expression with invasion, migration and EMT process of NSCLC, and Nef might reduce these processes by inhibiting ROCK1 expression.

According to the results of dependent relevance of ROCK1 and Nef, we provided evidence that Nef could interact with ROCK1 protein by molecular docking (**Figure 7A-D**). The results showed the binding free energy of Nef with AcROCK1 and BcROCK1 were -10.08 KJ/mol and -9.00 KJ/mol, respectively, which were higher than Y27632, a well-known ROCK1 inhibitor, with AcROCK1 (binding free energy: -7.1 KJ/mol) or BcROCK1 (binding free energy: -7.2 KJ/mol), suggesting Nef had stronger interaction with ROCK1 than Y27632. Besides, molecular docking also showed that the number of residues of ROCK1 interacted with

Nef were more than Y27632 (**Supplementary Figure 1E-F**). It suggested that Nef might block invasion, migration and EMT process through inhibiting ROCK1.

Discussion

A significant progress, including chemotherapy, radiotherapy, immunotherapy and tyrosine kinase inhibitors, has been made in the diagnosis and treatment of NSCLC in recent years, however the 5-year overall survival rate of patients with NSCLC has remained as low as 16%. The recurrence and tumor metastasis currently remain as the major obstacle in clinical cancer treatment (Pan et al. 2019), and it istherefore imperative to develop novel therapeutic agents against tumor invasion and motility to overcome the shortcomings of current treatment of lung cancer (Herbst et al. 2018). In this study, we illustrated the antitumor effects of Nef, a natural compound from green seed embryos of Lotus, on two NSCLC cells lines *in vitro*. Nef also decreased the tumor growth in *vivo*. We have demonstrated that Nef exerts cytotoxicity effects to the NSCLC cell lines in micromolar concentrations, induces apoptosis by regulating BCL2/BAX ratio. We also provided evidence that Nef can target ROCK1 protein through molecular docking and that Nef can significantly inhibit cell migration and invasion in would healing and transwell assays, and attenuate dysregulations of key oncogenic factors during EMT process in the NSCLC cells.

Previous evidences have shown the various pharmaceutical functions of Nef, including antitumor, anti-diabetic, anti-aging, anti-microbial, anti-thrombotic, anti-arrhythmic, anti-inflammatory and even anti-HIV (Marthandam Asokan et al. 2018). Our current study is in consistency with the recent literature and also enhances the attention on antitumor properties of Nef. For example, Nef was found to modulate JNK pathway activation, to inhibit cell proliferation and induce apoptosis in melanoma and prostate cancer (Nazim et al. 2020; Xie et al. 2020). Nef was also shown to possess antitumor activities via inhibiting cell invasion and EMT in hepatocellular carcinoma (Deng et al. 2017). The studies indicate the IC_{50} of Nef against LNCaP cells (prostate cancer), MCF-7 cells (breast cancer) and HCT8 cells (colon cancer) are 35 μM, 33.7 μM and 20.3 μM, respectively at 48 h (Erdogan and Turkekul 2020; Kadioglu et al. 2017). The IC_{50} value above were in a good agreement with H1299 cells, the NRAS mutant NSCLC (26.66 μM), but were much higher than Nef on A549 cells (14.34 μM), suggesting that A549 cells, which is KRAS mutant NSCLC, might be more sensitive to Nef treatment and it required further investigation. Besides, Nef treatment had more notable induction of ROS and BAX protein levels in A549 cells, whereas, the inhibitory in metastasis was similar in A549 and H1299 cells. NRAS mutation is rarely observed in NSCLC patients, and there are no approved treatments for NRAS-mutant NSCLC yet (Park et al. 2020). However, GATA2 knockdown also reduced the viability of cell lines carrying mutated loci functionally related to KRAS such as NRAS, NF1, EML4-ALK and EGFR (Kumar et al. 2012). Our results showed the inhibitory effect of Nef on H1299 cells, which indicated that inhibition of ROCK, downstream of GATA2, was also effective on NRAS mutant cells. Lung cancer is the major type of cancer death, and our current research together with others highlight the potential for developing Nef as a common antitumor drug in the wide range of cancer cell lines and in pre-clinical models.

We have analyzed the antitumor mechanism of Nef and identified a novel target of Nef, ROCK1, which is involved in cell invasion and EMT (Guo et al. 2019; Hu et al. 2019; Lin et al. 2018). In recent years, the dysregulation of the ROCK1/2 has been associated with increased metastasis and poor patient survival in several tumor types. ROCK1/2 play essential roles in regulating the cytoskeleton, and have been increasingly recognized as common targets for many types of cancer (de Sousa et al. 2020). Previous studies show that ROCK is involved in anchorage-independent growth and invasion regulation directly (Vigil et al. 2012). The application of fasudil (Fas), a ROCK1 inhibitor, shows the treatment value in cancers by reducing the invasion and metastasis of hepatocellular carcinoma (Hu et al. 2014; Shahbazi et al. 2020). Evidence is appearing that the inhibition of ROCK1, as an oncogene, can play the suppression role in tumor progression, especially in NSCLC (Hu et al. 2019; Xin et al. 2020). In KRAS-driven lung cancers, ROCK is controlled by KRAS-GATA2 interaction, and the application of Fas can inhibit tumor progression (Barbacid 2012; Kumar et al. 2012). All these results indicate the importance of developing new inhibitors of ROCK1 and exploring the therapeutic potential.

We have not only demonstrated the antitumor effect of Nef, but also showed that Nef targets ROCK1 with higher affinity than the existing ROCK inhibitor Y27632. As shown by molecular docking, 18 amino acid residues of AcROCK1 can interact with Nef, including Lys105, Gly85, Ala86, Phe87, Thr219, Ala215, Ser118, Gly218, Phe120, Ile82, Ala103, Glu154, Met156, Phe368, Met153, Val90, Gly83, and Asp216, and Nef forms hydrogen bonds with Lys105, Ser118 and Asp216. In addition, 17 amino acid residues of BcROCK1 can interact with Nef, including Asn163, Asp160, Phe368, Asn367, Asp369, Met153, Asp216, Ala215, Met156, Glu154, Ala103, Ile82, Gly83, Val90, Lys105, Lys200, and Asp202, and Nef can form hydrogen bonds with Asp369, Asp216, and Lys200. The numbers of amino acid residues in both AcROCK1 and BcROCK1 interacting with Y27632 are less than those of Nef. This may be related to the unique skeleton of Nef consisting of two benzylisoquinoline groups. It is suggested that Nef as the novel potential ROCK1 inhibitor has a higher binding affinity than Y27632. Furthermore, we show that Nef can decrease the expression of ROCK1 protein. We hypothesize that binding of Nef to ROCK1 may also influence the stability of ROCK1 leading to accelerated degradation and/or negative feedback of autoregulation, which are required to be further investigated in the subsequent studies, as currently there is lack of knowledge in specific pathway involving in ROCK1 degradation.

In summary, Nef as a natural compound isolated from Chinese medicine, has shown to play multiple anti-tumor roles, which include inhibiting cell viability, inducing apoptosis via regulation of ROS and BCL2/BAX balance, inhibiting invasion, migration and EMT process via onco-factors in NSCLC cells, in particular by targeting ROCK1 and decreasing ROCK1 protein contents. The study demonstrates the therapeutical potential of Nef as a novel ROCK1 inhibitor, which may find a wide application for common cancers. Our results also emphasized ROCK as the therapeutical targets of KRAS and NRAS mutant NSCLC. Further, the mechanism of ROCK1 inhibition, downstream targets and the connection between ROCK1 and EMT process should be also explored in the follow up studies. In addition to verify the therapeutic effects and mechanism of Nef on a wide range of NSCLC cell lines, we need to evaluate the safety and effectiveness of Nef *in vivo* in pre-clinical models and in human cell models, to provide a firm foundation of clinical trials and the application prospects of TCM in clinical cancer treatment.

Abbreviations

ANOVA
analysis of variance
BAX
BCL2 associated X protein
BCL2
B-cell lymphoma-2
DMSO
dimethyl sulfoxide
ELISA
enzyme linked immunosorbent assay
EMT
epithelial-to-mesenchymal transition
Fas
fasudil
IC_{50}
half maximal inhibitory concentration
Nef
Neferine
NSCLC
non-small cell lung cancer
OD
optical density
PBS
phosphate-buffered saline
qRT-qPCR
real-time quantitative polymerase chain reaction
ROS
reactive oxygen species
ROCK1
Rho-associated protein kinase 1
TCMs
traditional Chinese Medicines
TGF-β1
transforming growth factor-β1

Declarations

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

G. Yang and Y. Pan conceived the idea; P. Hu, P. Wan and B. Yan performed most of the research; Q. Xu and C. Liu conducted the molecular docking; Z. Wei, J. Xu and S. Liu analyzed the data; all authors contributed to the writing and revisions.

Ethics approval and consent to participate

Animal experiments were approved by the Review Board of Nanjing University of Chinese Medicine (Ethics number: 202011A002).

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Neferine (Nef) inhibits the cell viability of A549 and H1299 cells.

A. The molecular structure of Nef.

B. The effect of Nef on the cell viability of A549 and H1299 cells was determined by CCK8 assay. Cells were treated with Nef for 24 and 48 h. The data performed with three independent experiments and *asterisk* indicates a statistically significant difference.

C. The IC_{50} value of Nef against A549 and H1299 cells.

D. The cell viability of BEAS-2B cells treated with Nef. (*****$P<0.05$, ******$P<0.01$, *******$P<0.001$)

E. The xenograft tumor growth in *BALB/c* nude mice treated with Nef and Fas.

F. The mice weight of *BALB/c* nude mice treated with Nef and Fas.

Figure 2

Nef induces cell apoptosis in A549 and H1299 cells.

A. The cell apoptosis was determined by Annexin V/PI staining assay and detected by flow cytometry in A549 and H1299 cells treated with Nef for 48 h.

B. The ROS level was determined by DCFH-DA staining and detected by fluorescent microscope in A549 and H1299 cells treated with Nef for 48 h.

C-D. The protein expression of A549 and H1299 cells treated with Nef were detected by western blot. The expression of BAX and BCL2 were determined. GAPDH was used as loading control. The data performed with three independent experiments and *asterisk* indicates a statistically significant difference (*****$P<0.05$, ******$P<0.01$, *******$P<0.001$).

Figure 3

Nef suppresses the invasion and migration of A549 and H1299 cells.

A. A549 and H1299 cells were treated with Nef for 24 h, and the migration ability of cells was detected by wound healing assay.

B. A549 and H1299 cells were treated with Nef for 24 h, and the invasion ability of cells was detected by transwell assay.

C. The expression of MMP2 and MMP9 in A549 and H1299 cells treated with Nef for 24 h were detected by ELISA assay. The data performed with three independent experiments and *asterisk* indicates a statistically significant difference (*****$P<$ 0.05, ******$P<$ 0.01, *******$P<$ 0.001).

Figure 4

Nef reduces the EMT process of A549 and H1299 cells.

A. The morphology of H1299 cells treated with TGF-β1 for 12 and 24 h.

B-C. The EMT-related proteins including E-cadherin, N-cadherin and Vimentin were detected by western blot in A549 and H1299 cells treated with TGF-β1 or co-treated with TGF-β1 and Nef for 24 h and GAPDH was used as loading control (B). The EMT-related genes were detected by qRT-PCR (C). The data performed with three independent experiments and *asterisk* (compared to the group TGF-β1 treated alone) and *octothorpe* (compared to control group) indicates a statistically significant difference (*****$P<$ 0.05, ******$P<$ 0.01, *******$P<$ 0.001).

Figure 5

ROCK1 regulates cell invasion and migration in NSCLC cells.

A-B. The knockdown efficiency of *ROCK1* siRNA in A549 and H1299 cells were detected by western blot (A) and qRT-PCR (B).

C-D. The migration (C) and invasion (D) ability of A549 and H1299 cells transfected with *ROCK1* siRNA and treated with Nef was determined by transwell assay. The data performed with three independent experiments and *asterisk* indicates a statistically significant difference (*****$P<$ 0.05, ******$P<$ 0.01, *******$P<$ 0.001).

Figure 6

Nef inhibits the expression of ROCK1 in NSCLC cells.

A-B. The expression of ROCK1 protein were determined by western blot in A549 and H1299 cells treated with TGF-β1 (A) or Nef (B). GAPDH was used as loading control.

C. The expression of ROCK1 protein were determined by immunofluorescent assay in A549 and H1299 cells treated with Nef (total cells in each group> 100). The data performed with three independent experiments and *asterisk* indicates a statistically significant difference.

D. The ROCK1 protein expression in xenograft tumor in *BALB/c* mice treated with Nef and Fas. (*****$P<0.05$, ******$P<0.01$, *******$P<0.001$)

Figure 7

Nef interacts with ROCK1 protein.

A-D. Molecular docking examination of Nef and AcROCK1 and BcROCK1 protein. AcROCK1 binding mode (A) and 2D interaction map of Nef (C). BcROCK1 binding mode (B) and 2D interaction map of Nef (D).

Figure 8

The potential mechanism of Nef against NSCLC.

Supplementary Files

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