Tropical Journal of Pharmaceutical Research August 2019; 18 (8): 1615-1619
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i8.7

Original Research Article

Neferine induces apoptosis of pancreatic cancer cells through p38 MAPK/JNK activation

Tielan Feng[1], Hao Chen[2], Yaofang Peng[3], Xiaoming Sun[4]*
[1]Department of Operation Room, [2]Department of Cancer Center, [3]Department of Pharmacy, [4]Department of Nurse Department, The First hospital of Lanzhou University, Lanzhou City, Gansu Province 730000, China

For correspondence: **Email: XiaomingSunsdr@163.com; **Tel:** 0086-9318357027*

Sent for review: 16 May 2019 *Revised accepted: 30 July 2019*

Abstract

Purpose: *To investigate the functional role of neferine on pancreatic cancer (PC) cell apoptosis.*
Methods: *The pancreatic cell line, PANC-1 cells, was exposed with different concentration of neferine. CCK8 and flow cytometry (Cell counting kit-8) were carried out to detect cell proliferation and apoptosis. Protein expression was evaluated by western blot.*
Results: *Neferine suppressed cell viability and caused cell cycle arrest of pancreatic cells in a dose-dependent way. The effect of neferine on pancreatic cells was dependent on its ability to regulate the expression of cyclin E, cyclin D1, p21, cleaved caspase-3, cleaved PARP, Bcl-2 and Bax. In addition, neferine treatment induced the apoptosis of PANC-1 cells via promoting the activation of p38 MAPK/JNK signaling pathway.*
Conclusions: *Neferine inhibits cell viability and proliferation, and promotes apoptosis of PC cells by activating p38 MAPK/JNK signaling pathway. These results indicated the potential therapeutic effect of neferine in the treatment of PC.*

Keywords: *Neferine, Proliferation, Apoptosis, Pancreatic cancer, p38 MAPK/JNK*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Pancreatic cancer (PC) is considered the most fatal cancer in the world [1]. Although chemotherapy can minimally extend the life span of PC patients, at present, the 5-year survival rate is still lower than 4 % [2]. Thus, finding new therapeutic drugs is an urgent need to improve the survival of PC patients.

Traditional herbal medicines with low-toxicity and high anti-tumor activity are reported to be effective for the treatment of malignant cancers [3]. For example, Baicalein inhibits proliferation of bladder cancer cells [4]. Neferine, an alkaloid extracted from lotus seed, has shown great promise in the field of anti-tumor therapy [5]. Studies show that neferine promotes apoptosis and autophagy in human lung cancer cell line A549 by inhibiting the PI3K/Akt/mTOR signal pathway [6]. Neferine also induces cell cycle arrest and cell apoptosis to suppress liver cancer progression [7]. However, it is unclear if neferine has a therapeutic effect on PC.

JNK (c-Jun N-terminal kinase) is the member of

MAPK (mitogen-activated protein kinase) family, and involved in regulation of cancer cells apoptosis [8,9]. Neferine inhibits osteosarcoma cells proliferation in a p38 MAPK/JNK-dependent manner [10]. However, whether p38 MAPK/JNK is also involved in the regulatory effect of neferine on PC progression remains unknown. This study demonstrates the effect and identifies the molecular mechanism of neferine on PC cell regulation, suggesting the potential role of neferine in the treatment of PC.

EXPERIMENTAL

Cell culture

PANC-1 was acquired from American Type Cell Culture Collection (ATCC; Manassas, VA, USA), and cultured in RPMI (Roswell Park Memorial Institute) 1640 media (Gibco, Eggenstein, Germany) and incubated at 37 °C and 5% CO_2.

CCK8

PANC-1 cells, treated with 0, 5, 10, 20 μM neferine (Sigma Aldrich, St. Louis, MO, USA), were trypsinized and then seeded at 3×10^4 cells/well in 96-well plates for twenty four, forty eight, or seventy two h. The viability of PANC-1 cells was evaluated using a spectrophotometer at 490 nm (BioTek, Winooski, VT, USA).

Cell cycle and apoptosis

PANC-1 cells, treated with 0, 5, 10, or 20 μM neferine, were cultured in 6-well plates at 1×10^6 cells/well. After twenty four hours of incubation, cells were harvested and resuspended in 5 μL 100 μg/mL propidium iodide (PI) and 1 U/mL ribonuclease (Abcam, Cambridge, MA, USA), and then incubated in dark. Cell cycle was analyzed via a flow cytometer (Attune, Life Technologies, Darmstadt, Germany). For apoptosis, the cells were incubated with an additional 5 μL of FITC-conjugated annexin V and then evaluated by flow cytometry.

Western blot

Proteins were extracted from PANC-1 cells via RIPA buffer (KeyGen, Nanjin, China). Thirty micrograms of protein was subjected to SDS-PAGE and transferred onto PVDF membrane. Membranes were probed with appropriate antibodies, including rabbit anti-cyclin E, cyclin D1, p21 (1:1500), Bcl-2, Bax (1;2000), cleaved caspase-3, cleaved PARP (1:2500), p-Akt, Akt, JNK, p-JNK, p38, p-p38, and β-actin (1:3000) overnight after blocking with 5% BSA. All antibodies were purchased from Abcam. The PVDF membrane was probed with horseradish peroxidase-conjugated secondary antibodies (1:5000; Abcam). Immunoreactivity was detected using enhanced chemiluminescence (KeyGen, Nanjin, China).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was determined via SPSS software, version 19.0 (SPSS Inc, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for data comparison with statistical significance set at $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$.

RESULTS

Neferine decreased cell viability

Cell viability of PC cells treated with neferine was evaluated using the CCK8 assay. Treatment using neferine decreased viability when compared to control (Figure 1). Moreover, neferine treatment decreased cell viability in a dose-dependent way (Figure 1). Neferine also had a time-dependent inhibitory effect on cell viability (Figure 1).

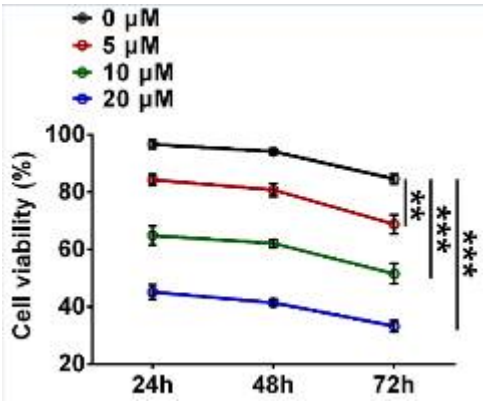

Figure 1: Influence of neferine on PANC-1 cell viability; n = 3; ** $p < 0.01$, *** $p < 0.001$ vs. control, respectively

Neferine promoted cell cycle arrest

As shown in Figure 2 A, treatment with various concentrations of neferine (5, 10, 20 μM) inhibited cell cycle progression and increased proportion of PANC-1 cells in G0/G1 phase when compared to control (0 μM neferine). Proportion of cells in G0/G1 phase with 5, 10, 20 μM neferine treatment were 86.14 ± 2.47, 90.89 ± 1.45, and 96.23 ± 1.21%, respectively. Neferine decreased expression of proteins involved in cell cycle regulation, cyclin E and cyclin D1, in a dose-dependent way. However, p21 expression

was increased following neferine treatment (Figure 2 B).

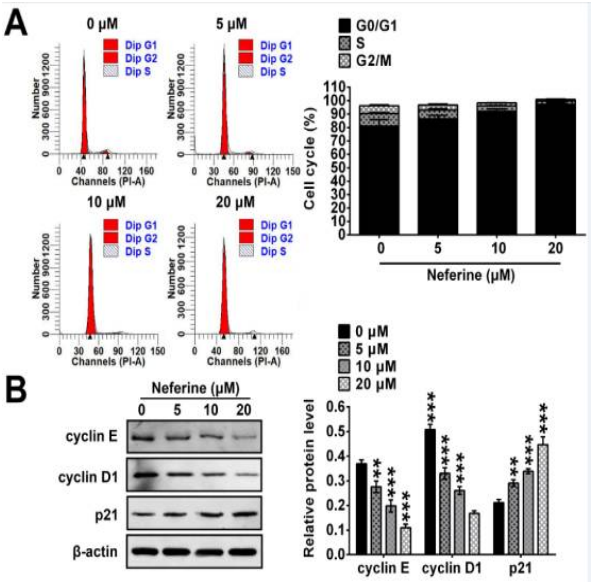

Figure 2: Influence of neferine on PANC-1 cell cycle. (A) Flow cytometric analysis of PANC-1 cell cycle progression following treatment with neferine. (B) Western blot analysis of protein expressions of cyclin E, cyclin D1, and p21 following neferine treatment of PANC-1 cells. ** $p < 0.01$, *** $p < 0.001$ vs. control

Neferine induced apoptosis

Treatment with various concentrations of neferine (5, 10, 20 μM) induced PANC-1 cell apoptosis when compared to control cells (0 μM) (Figure 3 A). The proportion of apoptotic of PANC-1 cells treated with 5, 10, or 20 μM neferine were 16.45 ± 2.71, 25.34 ± 2.16, and 33.67 ± 2.56 %. Neferine decreased apoptosis regulatory protein expression, Bcl-2. In contrast, Bax, cleaved caspase-3 and cleaved PARP expression were up-regulated via neferine treatment (Figure 3B).

Neferine promoted activation of p38 MAPK/JNK signal pathway

The underlying mechanism of neferine-induced regulation of PC progression was shown using western blot. As indicated in Figure 4, neferine treatment increased phosphorylation of p38 (p-p38) and phosphorylation of JNK (p-JNK) and increased the p-p38/p38 and p-JNK/JNK ratio in a dose-dependent way. However, neferine treatment had no significant effect on p-AKT or AKT expression or the p-AKT/AKT ratio, suggesting that neferine promoted activation of p38 MAPK/JNK signal pathway, but not AKT.

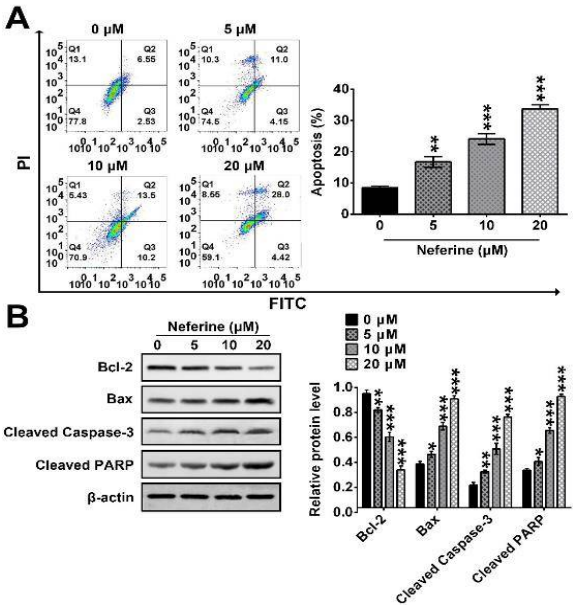

Figure 3: Influence of neferine on PANC-1 cell apoptosis. (A) Flow cytometric analysis of neferine-induced PANC-1 cell apoptosis. (B) Western blot analysis of Bcl, Bax, cleaved caspase-3 and cleaved PARP expression in PANC-1 cells following neferine treatment; ** $p < 0.01$, *** $p < 0.001$ vs. control

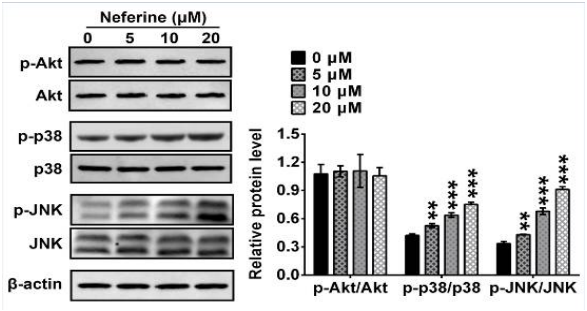

Figure 4: Influence of neferine on activation of p38 MAPK/JNK signaling. Western blot analysis of AKT, p38, JNK, p-AKT, p-p38, and p-JNK protein expression following treatment with neferine. ** $p < 0.01$, *** $p < 0.001$ vs. control

DISCUSSION

High rate of proliferation and invasive potential of PC makes it one of the most aggressive tumors [11]. Due to the absence of prominent symptoms during the early stages of PC [2], there is an urgent need to find more effective therapies for PC. Neferine shows anti-tumor capacity in various kinds of cancers including osteosarcoma [10], hepatocellular carcinoma [12], and lung cancer [6]. This study evaluated anti-tumor effect of neferine and discovered the underlying mechanism of neferine on PC cells.

In line with previous studies [5,13-15], neferine decreased cell viability and induced cell cycle

arrest of PANC-1 cells at G0/G1 phase. In particular, expression of cyclin-dependent kinase (CDK) regulators cyclin E and cyclin D1 decreased after neferine treatment. Cyclin D1 and cyclin E bind to and activate CDK2, CDK4, and CDK6 [16,17] to induce cell cycle progression. Moreover, neferine increased CDK inhibitor expression, p21, that suppresses cyclins/CDK complexes and blocks cell cycle progression [18].

In addition to the anti-proliferation effect of neferine on PC, this study also demonstrated that neferine induced apoptosis in PC, a common feature of neferine treatment of various cancers. Apoptosis is modulated by apopto-associated protein Bax [19], and anti-apoptotic associated protein Bcl-2 [20]. During neferine-induced apoptosis, down-regulation in Bcl-2 expression with up-regulation in Bax expression were found [21]. The activated forms of the caspase proteins cleaved PARP and caspase-3 are also involved in regulation of apoptosis [22]. Neferine increased cleaved PARP and cleaved caspase-3 expression, thus promoting apoptosis of PANC-1 cells.

Neferine promotes ovarian cancer cell apoptosis by activating the p38 MAPK/JNK signaling pathway [15], correlating with the findings with PC cells. In addition to the p38 MAPK/JNK signaling pathway, PI3K/AKT signaling pathway has also been indicated as regulator in the apoptosis of PC cells [23]. However, the present study did not detect activation of PI3K/AKT, as there was no significant effect on p-AKT or AKT expression. However, this is in line with a previous study [15]. Future investigation on how neferine activates the p38 MAPK/JNK pathway is required. Complicated molecular mechanisms are involved in apoptosis of PC cells. Thus, more studies are required to determine if neferine regulates autophagy through reactive oxygen species generation or other signaling pathways.

CONCLUSION

Neferine decreases PANC-1cell viability and promotes apoptosis. The protective influence of neferine against PC is associated with activation of p38 MAPK/JNK pathway. Therefore, neferine is a new potential therapeutic agent for PC.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests with regard to this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Tielan Feng designed all the experiments and revised the manuscript. Hao Chen performed the experiments. Yaofang Peng and Xiaoming Sun wrote the paper.

Open Access

REFERENCES

1. *Spadi R, Brusa F, Ponzetti A, Chiappino I, Birocco N, Ciuffreda L, Satolli MA. Current therapeutic strategies for advanced pancreatic cancer: A review for clinicians. World J Clin Oncol 2016; 7(1): 27-43.*
2. *Peng X-B, Wu G-J, Wang X-Y, Tang X-J, Gong L. Bryostatin I inhibits growth and proliferation of pancreatic cancer cells via suppression of NF-κB activation. Trop J Pharm Res 2016; 15(10): 2071.*
3. *Lee JW, Lee WB, Kim W, Min BI, Lee H, Cho SH. Traditional herbal medicine for cancer pain: a systematic review and meta-analysis. Complement Ther Med 2015; 23(2): 265-274.*
4. *Tang X, Chen J, Yu X, Tang L, Li Y, Xia J. Baicalein and U0126 suppress bladder cancer proliferation via MAPK signaling pathway. Trop J Pharm Res 2017; 16(2): 279.*
5. *Xue F, Liu Z, Xu J, Xu X, Chen X, Tian F. Neferine inhibits growth and migration of gastrointestinal stromal tumor cell line GIST-T1 by up-regulation of miR-449a. Biomed Pharmacother 2019; 109: 1951-1959.*
6. *Poornima P, Weng CF, Padma VV. Neferine from Nelumbo nucifera induces autophagy through the inhibition of PI3K/Akt/mTOR pathway and ROS hyper generation in A549 cells. Food Chem 2013; 141(4): 3598-3605.*
7. *Yoon JS, Kim HM, Yadunandam AK, Kim NH, Jung HA, Choi JS, Kim CY, Kim GD. Neferine isolated from Nelumbo nucifera enhances anti-cancer activities in Hep3B cells: molecular mechanisms of cell cycle arrest, ER stress induced apoptosis and anti-angiogenic response. Phytomedicine 2013; 20(11): 1013-1022.*
8. *Chen S-Y, Zheng K, Wang Z-q. Neuroprotective effects of Ellagic acid on Neonatal Hypoxic Brain Injury via Inhibition of Inflammatory Mediators and Down-*

regulation of JNK/p38 MAPK Activation. Tropical Journal of Pharmaceutical Research 2016; 15(2): 241.

9. *Ding S, Li H, Li X, Wang W, Du X, Dong G, Zhang P. Serum amyloid P down-regulates CCL-1 expression, and inhibits Ras/MAPK signaling and development of breast cancer. Tropical Journal of Pharmaceutical Research 2017; 16(9): 2089.*
10. *Zhang X, Liu Z, Xu B, Sun Z, Gong Y, Shao C. Neferine, an alkaloid ingredient in lotus seed embryo, inhibits proliferation of human osteosarcoma cells by promoting p38 MAPK-mediated p21 stabilization. Eur J Pharmacol 2012; 677(1-3): 47-54.*
11. *Hohne MW, Halatsch ME, Kahl GF, Weinel RJ. Frequent loss of expression of the potential tumor suppressor gene DCC in ductal pancreatic adenocarcinoma. Cancer Res 1992; 52(9): 2616-2619.*
12. *Poornima P, Quency RS, Padma VV. Neferine induces reactive oxygen species mediated intrinsic pathway of apoptosis in HepG2 cells. Food Chem 2013; 136(2): 659-667.*
13. *Priya LB, Baskaran R, Huang CY, Padma VV. Neferine ameliorates cardiomyoblast apoptosis induced by doxorubicin: possible role in modulating NADPH oxidase/ROS-mediated NFkappaB redox signaling cascade. Sci Rep 2017; 7(1): 12283.*
14. *Kalai Selvi S, Vinoth A, Varadharajan T, Weng CF, Vijaya Padma V. Neferine augments therapeutic efficacy of cisplatin through ROS- mediated non-canonical autophagy in human lung adenocarcinoma (A549 cells). Food Chem Toxicol 2017; 103(28-40.*
15. *Xu L, Zhang X, Li Y, Lu S, Lu S, Li J, Wang Y, Tian X, Wei JJ, Shao C et al. Neferine induces autophagy of human ovarian cancer cells via p38 MAPK/ JNK activation. Tumour Biol 2016; 37(7): 8721-8729.*
16. *Lim S, Kaldis P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development 2013; 140(15): 3079-3093.*
17. *Hengstschlager M, Braun K, Soucek T, Miloloza A, Hengstschlager-Ottnad E. Cyclin-dependent kinases at the G1-S transition of the mammalian cell cycle. Mutat Res 1999; 436(1): 1-9.*
18. *Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 2009; 9(6): 400-414.*
19. *Kandasamy K, Srinivasula SM, Alnemri ES, Thompson CB, Korsmeyer SJ, Bryant JL, Srivastava RK. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c and Smac/DIABLO release. Cancer Res 2003; 63(7): 1712-1721.*
20. *Lazou A, Iliodromitis EK, Cieslak D, Voskarides K, Mousikos S, Bofilis E, Kremastinos DT. Ischemic but not mechanical preconditioning attenuates ischemia/reperfusion induced myocardial apoptosis in anaesthetized rabbits: the role of Bcl-2 family proteins and ERK1/2. Apoptosis 2006; 11(12): 2195-2204.*
21. *Rybnikova E, Sitnik N, Gluschenko T, Tjulkova E, Samoilov MO. The preconditioning modified neuronal expression of apoptosis-related proteins of Bcl-2 superfamily following severe hypobaric hypoxia in rats. Brain Res 2006; 1089(1): 195-202.*
22. *Boulares AH, Yakovlev AG, Ivanova V, Stoica BA, Wang G, Iyer S, Smulson M. Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. J Biol Chem 1999; 274(33): 22932-22940.*
23. *Cheng HB, Bo Y, Shen WX, Ren XG, Tan JN, Jia ZR, Xu CL. Longikaurin E induces apoptosis of pancreatic cancer cells via modulation of the p38 and PI3K/AKT pathways by ROS. Naunyn Schmiedebergs Arch Pharmacol 2015; 388(6): 623-634.*