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PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330
Int J Hum Genet, 23(4): 224-231 (2023)
DOI: 10.31901/24566322.2023/23.04.824

Investigation of the Antitumour and Anticancer Effects of Neferine by Inducing Stress of Endoplasmic Reticulum in Human Cervical Cancer-Derived Cells (HeLa)

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KEYWORDS Alkaloid. Antitumour Efficacy. Apoptosis. Cervical Cancer Treatment. Therapeutic Agent

ABSTRACT The leading prevalent reason of cancer demise in women is cervical cancer. The endoplasmic reticulum (ER) is necessary in order to provide homeostasis of the cell as well could have a close relation with cancer. Neferine may exert anticancer effects by inducing apoptosis and stress of ER. The researchers observed neferine's antitumoural efficacy in human cervical cancer derived cells (HeLa) via ER stress. Neferine was applied to the HeLa cells, and a viability test was performed by the methyl thiazolyl tetrazolium proliferation (MTT) assay. Analysis of MTT assay demonstrated that neferine effectively blocked HeLa cells development in a dose-dependently. Neferine may promote ER stress and enhance anticancer efficacy in HeLa cells. Based on Western blot and quantitative real-time polymerase chain reaction (qPCR) analyses, the researchers found that neferine inhibited cervical cancer cells via the ER stress pathway. The results indicate that neferine is a potent anticancer therapeutic candidate.

INTRODUCTION

Among gynaecological cancers, cervical cancer is the major cause of demise. Treatment modalities of cancer are being developed in vivo and in vitro using new technologies (Siegel et al. 2020), and cytotoxic substances obtained from natural products, such as plants, have been found to take various parts in cancer treatment (Grothaus et al. 2010). Furthermore, natural compositions have been reported to possess therapeutic potential in alternative treatments of cervical cancer (Dasari et al. 2020). Many of such natural products work through different pathways to kill cancer cells, such as ER stress and ER stress-related apoptosis (Kim and Kim 2018).

The ER provides proper folding to create functional proteins. The tumour microenvironment could disturb the function of ER (protection of cell homeostasis), thereby causing the unfolded proteins gathering in the ER, well-known as stress of ER. This stress of ER may have a correlation to cancer. While stress of ER starts an unfolded protein response (UPR) to reconstitute homeostasis of the ER, sustained stress of ER may actuate the apoptotic pathway (Kim and Kim 2018).

The large amount of translation related with metabolism and proliferation of cancer cell may

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activate stress of ER and UPR. An active UPR creates a stress response via ER sensor proteins, such as the activation of transcription factor 6 (ATF-6), protein kinase R-like endoplasmic reticulum kinase (PERK) and serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme (IRE-1). On the other hand, if the stress is violent, constant and incorrigible, the UPR actuates an ER stress-related cell death pathway by up-regulating the downstream target pro-apoptotic C/EBP homologous protein (CHOP) via inducing transcription factor 4 (ATF4) (Maurel et al. 2015).

One of the bisbenzylisoquinoline alkaloid is neferine, obtained from Nelumbo nucifera and can produce anticancer effects via different mechanisms. For example, it can induce apoptosis and ER stress, actuating via various signalling cascades by the inducing of specific genes (Dasari et al. 2020). The researchers noticed the antitumoural effect of neferine in human cervical cancer derived cells via ER stress in this study.

Objectives

The researchers demonstrated the antitumour and anticancer efficacy of neferine and its potential utilization as an option in the treatment of cervical cancer.

METHODOLOGY

The effective dose of neferine in a cell culture with inhibitory concentration of 50% (IC_{50}) was found using methyl thiazolyl tetrazolium (MTT) assay to analyse the neferine's anticarcinogenic efficacy on stress of ER in human cervical cancer derived (HeLa) cells. This dose (IC_{50}) was then applied to HeLa cells and a qPCR was implemented to investigate gene expression levels; also, Western blotting was conducted to examine protein expression levels.

Cell Culture

In this study, a HeLa cell line and human embryonic kidney cell line (HEK293) were used as controls. After obtaining the HeLa cell line from the American Type Culture Collection (ATCC), it was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% penicillin/streptomycin antibiotics in a humidified incubator at 37 °C with 5% CO_2. When the cells covered 80 to 90% of the flask surface, they were passaged and stored.

MTT Proliferation Assay

The viability and effective dose (IC_{50}) of neferine on HeLa cells were measured by a standard method, the MTT proliferation assay. The HeLa cells were seeded with 100 µl fresh culture medium per well in 96-well plates (~4.5-5x10^3 cells). After the cells were cultured for 48 h, the neferine was applied to the wells at concentrations of 5 µM, 10 µM, 12.5 µM, 20 µM, 22.5 µM, 25 µM, 30 µM, and 50 µM at adjusted doses for 24, 48, and 72 h. 10 µl of a 12mM MTT solution (Sigma-Aldrich, Germany) was applied to the neferine-treated and untreated control cells and incubated at 37°C for 4 hours. The medium was then eliminated in a dark cell culture room.

DMSO (50 µl) was added to the wells, which were gently shaken for 20 min to deploy the crystal blue melts. The absorbance at 575 nm was recorded using the Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the IC_{50} dose of neferine was determined to be 20 µM.

The Application of IC50 Value to Cells, Total RNA Extraction and Synthesis cDNA

The HeLa and HEK293 cells were again treated 48 hours after seeding with the IC_{50} dose (20 µM) of neferine in a cell culture medium. All cell groups, including the control group, were subjected to the classical RNA isolation procedure by applying the TRIzol (Sigma-Aldrich), chloroform and isoamyl alcohol method. The RNA pellet was precipitated with 75% absolute ethanol (96%, v/v) and dissolved in nuclease-free water. cDNAs were synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) of total RNA equalized to 1µg according to the manufacturer's instructions.

qPCR

A qPCR analysis was applied for all genes in triplicate with the QuantStudio™3 Real Time PCR system (Applied Biosystems).

The nucleotide sequences of primer pairs ER degradation-enhancing alpha-mannosidase-like protein 1 (EDEM1), activating transcription factor 3 (ATF3), ATF4, ATF6, X-box binding protein-1 (XBP-1), CHOP, glucose-regulated protein 78 (GRP78) and IRE1 in the stress of ER pathway used for quantitative gene expression are given in Table 2. A thermal profile followed by melting curve analysis steps was performed for 15 minutes at 95 °C, 40 cycles at 95 °C for 15 seconds, 56-60°C for 30 seconds and 72 °C for 15 seconds. The comparative ΔCT method and glyceraldehyde 3-phosphate dehydrogenase (GAPHD) as housekeeping gene were used to compute the notional quantification of gene expression.

Western Blotting

Dose-treated (IC_{50}) and untreated (control) HeLa and HEK293 (neferine-treated and untreated) cells were homogenized in an RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% nonidet P-40) containing a protease inhibitor cocktail (Sigma-Aldrich). The proteins were discreted by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 6-10% acrylamide/bisacrylamide gels and transmited onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were analyzed with the following primary ER stress pathway antibodies: PERK (D11A8) Rabbit mAb 5683, PDI (C81H6) Rabbit mAb 3501, BIP (C50B12) Rabbit mAb 3177, GRP78/HSPA5 antibody, Ero1-La antibody 3264, IRE1α (14C10) Rabbit mAb 3294, Calnexin (CANX) (C5C9) Rabbit mAb 2679, CHOP (L63F7) Mouse mAb 2895 and HRP-linked antibody (Cell Signaling Technology).

The presence of the antigens used was determined utilizing an enhanced chemiluminescence detection kit (Amersham Bioscience, Piscataway, NJ, USA). Their Western blot profiles were exposure recorded and captured on a RAS-4000 image reader (Fujifilm, Japan). Band profiles were further examined utilizing the Image J (Bethesda, MD, USA) imaging and the analysis program.

Statistical Analysis

Statistical analysis was performed with IBM SPSS version 21.0. Based on the Student t-test results, comparisons were made among the treated and control groups for the genes analysed. The levels of ATF4, ATF6, ATF3, EDEM1, CHOP, XBP1, GRP78 and IRE1 expressions were analysed by the $2^{-\Delta\Delta Ct}$ method developed by Livak and Schmittgen (2001).

RESULTS

Neferine-inhibited Cell Proliferation in HeLa Cells

Neferine inhibits the proliferation of HeLa cell lines in vitro via stress of ER. The different concentrations of neferine (5, 10, 12.5, 15, 20, 22.5, 25, 30 and 50 μM) for 24, 48 and 72 h were applied on HeLa cells to analyse the impact of neferine on cell viability and the cell viability was interpreted by MTT assay. The outcomes indicate that neferine significantly blocked HeLa cells growth in a dose- dependently (Table 1). The results also show that neferine clearly exhibits an anti-proliferative effect on HeLa cells. The IC_{50} dose of neferine was determined as 20 μM for 48 hours using the results of this analysis, indicating that neferine has a strong effect against the proliferation of HeLa cells at this dose.

Neferine-induced ER Stress in HeLa Cells

The expression analysis of genes associated with stress of ER was executed by a real-time PCR analysis involving the treated groups of HeLa and HEK293 cells. The researchers evaluated the levels of ATF3, ATF4, ATF6, EDEM-1, CHOP, XBP-1, GRP78 and IRE-1 genes to analyse ER stress. The real-time PCR evaluation demonstrated that neferine increased the mRNA levels of ATF4, ATF3, EDEM-1, CHOP, XBP-1, GRP78 and ATF6 but decreased IRE1 in a dose-dependently in HeLa cells. These data propound that neferine could trigger ER stress in HeLa cells.

At this point, to get the true fold change, the log base of this value was taken to even out the scales of up-regulated and down-regulated genes. Otherwise, the up-regulated genes have a scale of 2–infinity, while the down-regulated ones have a scale of 0–2. An increase of 8 times in ATF4, 6 times in ATF3, 4 times in EDEM-1, 9 times in GRP78, and 7 times in CHOP and XBP1

Table 1: Results of the MTT Assay. MTT assays were performed six times for each sample.The mean values and standard deviations were given in the table

	Neferine Administration Durations					
	24 hour		*48 hour*		*72 hour*	
Concentration	*Mean*	*% Viability*	*Mean*	*% Viability*	*Mean*	*% Viability*
0 (Control)	2.89±0.12	100	2.21±0.30	100	1.89±0.04	100
5	2.74±0.22	94.71	2.01±0.42	90.70	1.22±0.26	64.45
10	2.53±0.14	87.44	1.99±0.18	90.12	1.09±0.10	57.62
12.5	2.46±0.19	85.11	1.99±0.08	90.07	1.07±0.17	56.50
15	2.47±0.18	85.61	1.89±0.32	85.50	1.00±0.23	53.17
20	2.49±0.16	86.09	1.09±0.29	49.79	1.11±0.30	58.96
22.5	2.42±0.11	83.85	1.06±0.19	48.15	0.97±0.28	51.28
25	2.41±0.16	83.56	0.98±0.03	44.60	0.79±0.22	41.97
30	2.24±0.19	77.58	1.06±0.00	48.21	0.75±0.03	39.95
50	1.54±0.15	53.49	1.01±0.06	45.67	0.81±0.08	42.74

were observed. The values found were all significant, $P<0.05$ (Table 2).

ER stress has been observed in cells treated with neferine in cervical carcinomas. Next, Western blot evaluation was performed to identify the impact of neferine on GRP78; PERK; Protein Disulfide-isomerase (PDI); IRE1; ER 0xidoreductase 1 alpha (ERO1); CANX, a molecular sign of ER stress; and CHOP, a protein related with ER stress-induced apoptosis (Fig. 1). The analysis demonstrated that neferine increased the protein levels of GRP78, ERO1, CANX and CHOP and decreased PERK, PDI and IRE1 in a dose-dependently in HeLa cells. The PERK and IRE1 reduction detected in this present study may potentially result in the inhibition of tumour development. A further finding of this study is that the increase in GRP78, CHOP and ERO1 levels may support the inhibition of cancer cell proliferation via ER stress-induced apoptosis.

DISCUSSION

Cervical cancer is a prominent cause of cancer demises worldwide, according to Cancer Statistics 2021. Hence, new anticancer agents and therapies that are more efficient and have less side effects must be developed for treating cervical cancer. A wide variety of plant-derived bioactive agents have shown antitumour activity (Bishayee et al. 2022; Dennis et al. 2009), while natural compounds or their bioactive agents have been reported to increase apoptosis and decrease resistance to chemotherapies by inducing ER stress (Kim and Kim 2018). Neferine has been studied as an anticancer therapy for many different cancers and has been shown to have anticancer effects in HeLa cells (Dasari et al. 2020). Neferine dominates anticancer activities, including autophagy, cell cycle arrest, stress

Table 2: Expression results of genes associated with ER stress pathway analysed by delta CT method

	Mean CT		*Standard Deviation_CT*				
Genes	*Mean target*	*Mean control*	*Standard deviation target*	*Standard deviation control*	*P value*	*Fold change 2^-ΔCT*	*Fold change log2*
ATF4	-2.88245	4.9665	0.881912	0.2235	0.000117	230.5516	7.848946
ATF6	6.837457	8.041	2.090734	0.042	0.375249	2.303046	1.203543
ATF3	-0.111	6.1	0.905401	0.906	0.0011	74.07938	6.211
EDEM1	4.366579	8.144	1.235334	0.252	0.006564	13.71251	3.777421
CHOP	-1.04019	6.1435	1.04787	0.2785	0.000329	145.3804	7.183689
XBP1	-0.45861	6.3295	0.228908	1.0145	0.000349	110.5156	6.788106
GRP78	-2.68247	6.839	0.394768	0.859	0.000063	734.9357	9.521474
IRE1	8.388102	9.1295	0.151278	0.5915	0.103269	1.671796	0.741398

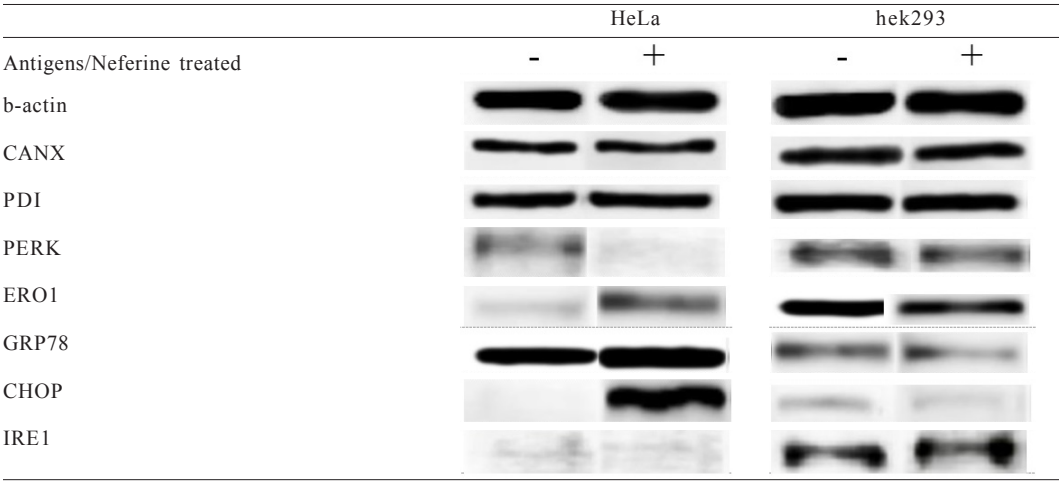

Fig. 1. Western blot analysis of the levels of GRP78, ERO1, IRE1, CANX, PDI, PERK, and CHOP after Neferine treatment

of ER, apoptosis and anti-angiogenesis (Bishayee et al. 2022; Yoon et al. 2013).

In this research, Western blotting was used to investigate neferine-activated ER stress, and increased expression of ER stress markers like ERO1, GRP78, CANX and CHOP was observed. On the other hand, neferine decreased the expression of CHOP, GRP78 ,ERO1 in the control group. Furthermore, the mRNA expression of GRP78, EDEM 1, XBP1, CHOP, ATF 3 and ATF 4 were more elevated than the control group.

In line with previous literature, the present study revealed a decrease in ER stress response molecules (PERK, IRE1 and ATF6) with Western blotting and a decrease of IRE1 only in a real-time PCR analysis. These findings seem to show that the stress of ER is violent and incorrigible. In addition, the significant increase of CHOP levels reported may support the inhibition of cancer cell proliferation via ER stress-induced apoptosis.

Neferine is a plant-derived bioactive agent. Dasari et al. (2020) showed that neferine is an antitumoural agent for cervical cancer cells via increasing apoptosis and autophagy. Maneenet et al. (2021) demonstrated the cytotoxic effect of neferine in HeLa cells by providing apoptosis with caspase 3 activation and Bcl-2 inhibition.

The ER has been reported to be helpful in maintaining cell homeostasis and cell viability (Ozcan and Tabas 2012; Scott 2020). However, hostile situations in the tumour microenvironment may influence the protein folding capacity, thereby triggering stress of ER (Bettigole and Glimcher 2015). An increase of misfolded proteins induces the UPR to maintain homeostasis in the ER. The UPR drives various adaptable and survival procedures (Chevet et al. 2015; Davies et al. 2008); nevertheless, when stress of ER is intense and constant; the UPR mediators that ensure viability may initiate apoptosis and ultimately terminate damaged cells (Kim et al. 2012; Tabas and Ron 2011). The relationship between the UPR and tumour development has been widely discussed in recent studies (Pytel et al. 2016). It is still debated whether the UPR increases or decreases tumor development (Scott 2020). Moreover, increases in UPR-connected factors have been found in patients with various cancer species, and their excessive increase is related to poor prognosis and treatment resistance (Dalton et al. 2013).

Studies have identified the role of the PERK and IRE1α pathways in tumour development in vivo. For example, the incorporation of malignant cells lacking PERK or IRE1α in mice caused tumour growth arrest despite the increased sensibility of cancer cells to ER stress upregulating agents (Bi et al. 2005). In response, different preclinical disease models have been treated by tar-

geting PERK or IRE1α signalling using specific molecule inhibitors in vivo (Cross et al. 2012; Ghosh et al. 2014; Tang et al. 2014). The decrement of PERK signalling in malignant cells makes them highly vulnerable to cell demise induced after exposure to hypoxia, nutrient deprivation and DNA damage (Maas and Diehl 2015). Thus, blocking PERK results in cancer cell apoptosis and obvious antitumour influences (Szegezdi et al. 2006). On the other hand, there are studies showing that IRE1 and PERK inhibition or deficiency increase tumor cell growth and development (Harrington et al. 2015; Pytel et al. 2016). In this research, neferine decreased IRE1α and PERK protein levels in HeLa cells (Fig. 1). This reduction was not observed in the control group. Neferine reduced the mRNA expression of IRE1α in both groups, but the influence on IRE1α protein level was not observed in the control group.

The present study investigated neferine activated stress of ER and found the increased ER stress markers expression, like ERO1, GRP78, CHOP and CANX through Western blot analysis. On the other hand, neferine decreased CHOP, GRP78 and ERO1 expression in the control group. Furthermore, the mRNA expression of GRP78, EDEM 1, XBP1, CHOP, ATF 3 and ATF 4 was expressively higher than that of the control group.

As previous studies have reported, the researchers found a decrease in ER stress response molecules (PERK, IRE1 and ATF6) through Western blotting and a decrease of IRE1 only by real-time PCR analysis (Scott 2020). These findings could show that the stress of ER is severe and cannot be repaired. In addition, the significant increase of CHOP levels may support the inhibition of cancer cell proliferation via ER stress-induced apoptosis.

The anticancer activity of anticancer agents is frequently associated with their capability to trigger tumour cell apoptosis. Many studies have reported that stress of ER is related with apoptosis (Pistritto et al. 2016; Al-Bahlani et al. 2017), which is responsible for cancer cell death after treatment. The ER induces and regulates apoptosis; increased apoptosis by ER stress can be used as an anticancer treatment (Fang et al. 2021; Kim et al. 2004; Zong et al. 2003).

From this study's results, it can be concluded indirectly that stress of ER act a role in the neferine-activated apoptosis of cervical cancer cells. The study showed that neferine can activate ER-associated apoptosis in HeLa cells based on the following findings: neferine meaningfully raised the ER stress markers expression like GRP78, CHOP, ERO1; neferine strongly increased the expression of CHOP and ATF4, important mediators of ER stress-activated apoptosis; and neferine decreased the expression of prosurvival pathways like IRE-1 and PERK in HeLa cells. More studies are needed for the anticancer and antitumour effects of neferine on cervical cancer cells.

CONCLUSION

Neferine, a herbal alkaloid, reduces cell vitality in HeLa cells and can activate antitumour and anticancer efficiency by inducting apoptosis through ER stress. Because neferine can activate apoptosis through raising stress of ER markers in cervical cancer cells, it can potentially be utilized as a beneficial factor in the pharmacological therapy of cervical cancer.

RECOMMENDATIONS

More studies should be done to investigate the pharmacological and therapeutic effects of neferine in cervical cancer cells. The effects of neferine on different cervical cancer cell cultures and its different pathways in anticancer mechanisms should also be investigated.

FOOTNOTES

The antioxidant, anti-inflammatory, antiproliferative and anti-angiogenic effects of neferine support its anticancer effect. Neferine has been investigated in the treatment of gastrointestinal, breast and liver cancers as well as gynaecological cancers, such as ovarian and cervical cancers.

ABBREVIATIONS

ATF3: Activating transcription factor 3
ATF4: Activating transcription factor 4
ATF-6: Activating transcription factor 6
CANX: Calnexin
CHOP: C/EBP homologous protein
DMEM: Dulbecco's modified Eagle's medium

EDEM1:Endoplasmic reticulum degradation-enchancing alpha-mannosidase-like 1

ER: Endoplasmic reticulum

ERO1: Endoplasmic reticulum Oxidoreductase1 alpha

FBS: Fetal bovine serum

GAPDH: Glyceric acid phosphate dehydrogenase

GRP78 : Glucose regulated protein 78

HEK293: Human embryonic kidney cell line

HeLa: Human cervical cancer-derived cells

IC_{50}: Inhibitory concentration %50

IRE-1: Serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme

MTT: Methyl-thiazolyl-tetrazolium proliferation assay

UPR : Unfolded protein response

PERK: Proteins like protein kinase R-like endoplasmic reticulum kinase

PDI: Protein disulfide isomerase

QPCR: Quantitative real-time polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

XBP1: X-box binding protein1

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Paper received for publication in April, 2022
Paper accepted for publication in June, 2022