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Protective Effects of Relaxin 2 (RLXH2) against Hypoxia-Induced Oxidative Damage and Cell Death via Activation of The Nrf2/HO-1 Signalling Pathway in Gastric Cancer Cells

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Abstract

Objective: This study aims to investigate the potential role of relaxin, a peptide hormone, in preventing cellular deterioration and death in gastric carcinoma cells under hypoxic conditions. It explores the effects of recombinant relaxin 2 (RLXH2) on growth, cell differentiation, invasive potential, and oxidative damage in these cells.

Materials and Methods: In this experimental study, the NCI-N87 cell line was cultured under normal conditions and then subjected to hypoxia using cobalt chloride ($CoCl_2$). The cells were treated with RLXH2, and various assays were performed to assess cellular deterioration, death, and oxidative stress. Western blot and quantitative real time polymerase chain reaction (qRT-PCR) were used to measure the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and HO-1, and the translocation of Nrf2 to the nucleus was confirmed through Western blot analysis.

Results: This study demonstrates, for the first time, that RLXH2 significantly reduces the formation of reactive oxygen species (ROS) and the release of lactate dehydrogenase (LDH) in gastric cancer cells under hypoxic conditions. RLXH2 also enhances the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), leading to a decrease in hypoxia-induced oxidative damage. RLXH2 promotes the translocation of Nrf2 to the nucleus, resulting in HO-1 expression.

Conclusion: Our findings suggest that RLXH2 plays a significant protective role against hypoxia-induced oxidative damage in gastric carcinoma cells through the Nrf2/HO-1 signalling pathway. This research contributes to a better understanding of the potential therapeutic applications of RLXH2 in gastric cancer treatment.

Keywords: Gastric Cancer, HO-1, Hypoxia, Nrf2, Relaxin

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Introduction

Relaxin, a peptide hormone, was initially found to be associated with pregnancy (1). Over time, relaxin was determined to carry out diverse functions in different diseases like cancer, heart failure, and diabetes (2-4). Three types of relaxin peptides are expressed in humans-relaxin 1 (RLXH1), relaxin 2 (RLXH2), and relaxin 3 (RLN3). Each type performs a different function (5). RLXH1 and RLXH2 act via binding to their respective receptors, RXFP1 and RXFP2, respectively. These receptors are a unique type of G-protein coupled receptors that consist of a large ectodomain and an N-terminal module. RLXH2 is a pleiotropic peptide hormone that is overexpressed in various types of cancers (6). RLXH2 helps in proliferation, invasiveness, and metastasis in cancer cells.

Hypoxia is a commonly observed phenomenon in different cancers, which causes activation of hypoxia-associated pathways in these cells (7, 8). Cancerous cells adapt to this hypoxic environment by stimulating metabolic alterations and angiogenesis in order to survive the hypoxic conditions. Response of cancerous cells to hypoxia leads to an aggressive phenotypic behaviour, chemotherapy resistance, and poor clinical outcomes (9). Hypoxia is a well-known phenomenon in different types of cancers like gastric cancer (10). The hypoxic environment within cells is associated with increased production of reactive oxygen species (ROS) (11). The enhanced ROS production is responsible for cellular injuries and often results in cell death (12). Therefore, to prevent hypoxia-associated injuries and death, cells often activate responses by scavenging ROS (13).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that plays a crucial role in cellular defence against oxidative stress and xenobiotic insults (14). Its activation is essential for maintaining cellular homeostasis, and dysregulation of Nrf2 signalling has been implicated in various cancers (15). Aberrant activation of the Nrf2 pathway has been observed in many cancer types, including lung, breast, prostate, colorectal, and liver cancers (16). The persistent activation of Nrf2 in cancer cells is associated with several tumour-promoting effects (17). Nrf2 activation enhances the cellular antioxidant capacity by upregulating

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the expression of genes that encode antioxidant enzymes (18). This increased antioxidant defence allows cancer cells to counteract oxidative stress and maintain a redox balance, which promotes cell survival and resistance to chemotherapy and radiotherapy.

The involvement of Nrf2 in cancer extends beyond its effects on oxidative stress, cell survival, and metabolism. Nrf2 is implicated in promoting cancer cell invasion, metastasis, and resistance to anti-cancer therapies. Nrf2 can modulate the expression of genes involved in epithelial-mesenchymal transition, extracellular matrix remodelling, angiogenesis, and drug efflux transporters, and it contributes to cancer cell migration, invasion, and therapy resistance.

In summary, while Nrf2 activation serves as a critical defence mechanism against oxidative stress and xenobiotic insults in normal cells, dysregulated Nrf2 signalling in cancers can confer numerous advantages to tumour cells, including increased antioxidant capacity, enhanced cell survival and proliferation, metabolic reprogramming, and resistance to therapies. Therefore, targeting the Nrf2 pathway is a potential therapeutic strategy to overcome therapy resistance and improve cancer treatment outcomes. The present work aims to explore the protective role of RLXH2 against hypoxia-associated cellular damage and death in gastric cancer cells.

Materials and Methods

The study has been approved by the Research Review and Ethics Board (RREB) of Shidong Hospital (SH/2018/0029).

Cell culture, treatments, and transient transfection

In this experimental study, the NCI-N87 gastric carcinoma cell line (ATCC) was grown in DMEM medium with 5% FBS and culture conditions of 37°C and 5% CO_2. The cells were grown in culture for 24 hours and either left untreated or treated with RLXH2 (Abcam, MA, USA) in 12-well plates. For the hypoxia-related experiments, we grew the cells in DMEM without serum and glucose, and treated them with 200 µM cobalt chloride ($CoCl_2$, Sigma-Aldrich, USA) for 12 or 24 hours with or without RLXH2 (15 nmol/l, Sigma-Aldrich, USA). Inhibition and activation of the Nrf2 pathway was carried out chemically. The NCI-N87 cells were grown overnight and treated with trigonelline (5 nM, Sigma-Aldrich, USA) or 100 µM tert-butylhydroquinone (tBHQ, Sigma-Aldrich, USA), respectively, according to a previously published protocol (19). Chemical inhibition of HO-1 was carried out by growing NCI-N87 cells for 24 hours and subsequently treating them with 2 µM zinc protoporphyrin IX (ZnPPIX, Sigma-Aldrich, USA) for 12 hours. siRNA technology was used to silence Nrf2. For that purpose, NCI-N87 cells were grown for 24 hours and then transiently transfected with 1 µM Nrf2-siRNA (Santa Cruz, Biotechnology, USA) using calcium phosphate (Sigma-Aldrich, USA).

Lactate dehydrogenase leakage assay

The gastric carcinoma cells were grown in a 12-well plate (2 ml medium/well) for 24 hours. The cells were treated under hypoxic only or hypoxic with RLXH2 (15 nmol/L) conditions for 12 hours. Then, 300 µl of the medium was removed and analysed for lactate dehydrogenase (LDH) activity using an LDH Cytotoxicity Assay kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Antioxidant assays

The gastric carcinoma cells were grown in a 12-well plate (5×10^5 per well) for 24 hours. The cells were treated under hypoxic only or hypoxic with RLXH2 (15 nmol/L) conditions for 12 hours. The cells were then washed three times in wash buffer (PBS with 0.05 mM EDTA), followed by sonication to disrupt the cells. Centrifugation at 3000 rpm for 10 minutes was carried out to separate the supernatant from the cell lysate. The resultant supernatant was assayed for superoxide dismutase (SOD, ThermoFisher Scientific, USA, cat. no. EIASODC), glutathione peroxidase (GPX, Sunlong, China, cat.no. SL2786Hu), and catalase (CAT, Biocompare, USA, cat. no. ELH-CAT-1) activities by following the manufacturers' instructions. The standards, controls, and working solutions were prepared according to the manufacturer's instructions in the ELISA kit.

Reactive oxygen species measurement

Gastric carcinoma cells were cultured in a 96-well plate (0.42×10^5 cells/well) for 24 hours. For analysis purposes, the treated cells were washed with 1X wash buffer followed by the addition of diluted DCFDA solution (100 µl/well) at 37°C. After 30 minutes of incubation, the DCFDA solution was aspirated and the cells underwent hypoxic treatment alone or together with RLXH2 for 12 hours. Finally, the amount of fluorescence in the cells was assessed.

Extraction of RNA and quantitative real time polymerase chain reaction

Total RNA was extracted from cultured cells using TRIzol-T Reagent (ThermoFisher Scientific, USA) followed by cDNA strand synthesis. The extracted cDNA was subjected to quantitative real time polymerase chain reaction (qRT-PCR) using primers against *Nrf2* and *HO-1*. *β-actin* was used for loading control purposes. The following primer sequences were used:

Nrf2-

F: 5′-CCTCAACTATAGCGATGCTGAATCT-3′
R: 5′-AGGAGTTGGGCATGAGTGAGTAG-3′

HO-1-

F: 5′-GGGCCAGCAACAAAGTG-3′
R: 5′-AGTGTAAGGACCCATCGGAGAA-3′

β-actin-

F: 5′-AGGCATCCTCACCCTGAAGTA-3′
R: 5′-CACACGCAGCTCATTGTAGA-3′

Protein extraction

Cell lysate was prepared from the NCI-N87 cells using NP-40 lysis buffer. In order to inhibit proteolysis, the Halt

Protease Inhibitor Cocktail (ThermoFisher Scientific, USA) was incorporated into the lysis buffer. The cell lysate was subjected to centrifugation at 3000 rpm for 10 minutes, and the supernatant was collected. The protein concentration in the obtained lysate was determined by the Bradford assay method.

Nuclear fraction preparation

A Nuclear Extraction kit (ThermoFisher Scientific, USA) was used to prepare nuclear extracts of the NCI-N87 cells and the Bradfords assay was used to determine protein concentrations. Western blot analysis was used to determine the purity of the nuclear fractions using specific antibodies according to a previously published protocol (20).

Western blot analysis

Protein samples were prepared according to a previously published protocol (21). The proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and later transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated overnight with the following primary antibodies: anti-Nrf2 (CST, #12721, 1:1000), anti-HO-1 (CST, #5853, 1:1000), anti-β actin (Abcam, #ab8227, 1:1000), anti-GAPDH (Sigma-Aldrich, #G8795, 1:1000), and anti-histone 4 (H4) (CST, #2592, 1:1000). The membranes were rinsed with PBS and further incubated with fluorescent-labelled secondary antibodies (LI-COR, fluorescent anti-mouse IRDye 680, 1:20 000 and LI-COR, anti-rabbit IRDye 800, 1:10 000). The LI-COR system (Biosciences, Lincoln, NE, USA) was used for secondary detection.

Quantification of immunoblots

Protein band quantification was performed using a LI-COR scanner. In order to create a standard plot, fluorescent spots from various concentrations of fluorescent-labelled secondary antibodies were measured. The fluorescence levels of the individual blot bands were measured and compared to standard plot to quantify the protein bands on the immunoblots.

Apoptotic assay

The gastric carcinoma cells were grown in 96-well plates for 24 hours. Then, the cells were either treated under hypoxic alone or hypoxic plus RLXH2 conditions for 12 hours. A Cell Death Detection ELISAPLUS kit (CELLDETH-RO Roche, USA) was used to determine apoptosis according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (version 9, GraphPad Software, Inc. USA). The data were analysed using One-way ANOVA followed by Duncan's test for multiple comparisons. The experimental values are presented as mean ± standard error of the mean with a statistical significance of $P<0.05$.

Results

Impact of relaxin 2 on lactate dehydrogenase and reactive oxygen species

Figure 1A shows successful induction of hypoxia after treatment with $CoCl_2$. The hypoxia resulted in membrane damage to the NCI-N87 cells, which was confirmed by LDH release. However, RLXH2 treatment significantly prevented LDH release and excessive ROS production from the hypoxia-damaged NCI-N87 cells (Fig.1B, C).

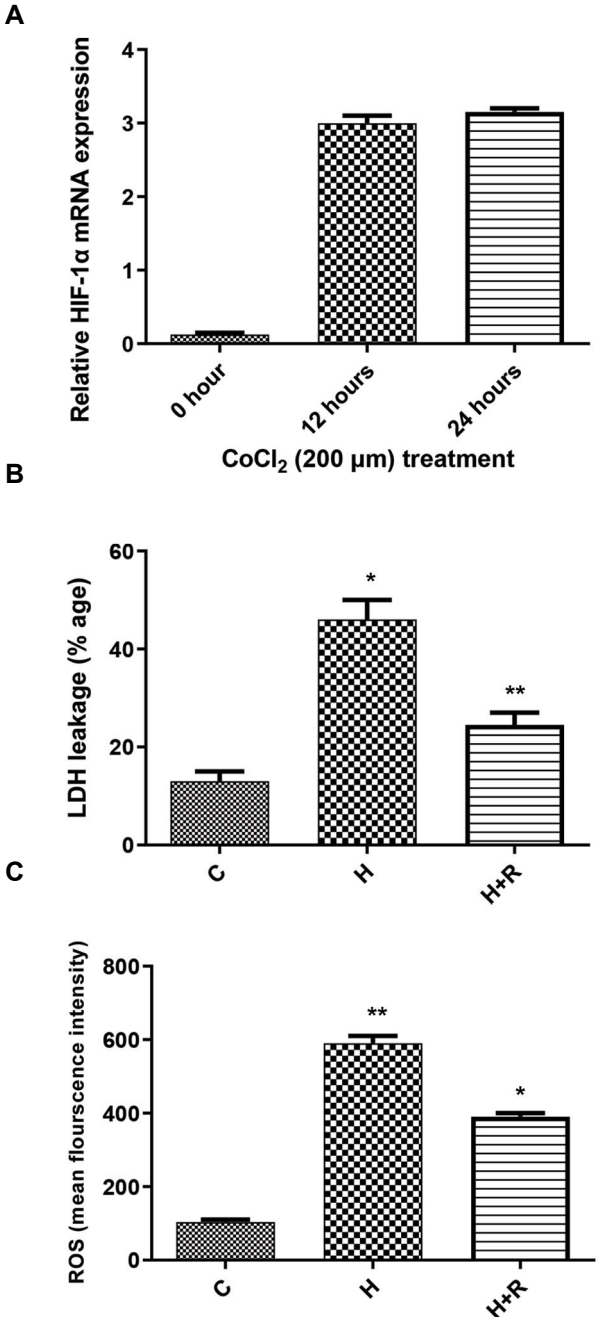


Fig.1: Impact of relaxin 2 (RLXH2) on lactate dehydrogenase (LDH) release and reactive oxygen species (ROS) formation. **A.** Induction of HIF-1α expression after hypoxia treatment for 12 and 24 hours. **B.** RLXH2 significantly decreased LDH release from the NCI-N87 cells in the hypoxia (H) group compared to the control (C) group ($P<0.05$). A $P<0.01$ was obtained from the hypoxia plus RLXH2 treatment group (H+R) compared to the H group. **C.** RLXH2 significantly decreased ROS production in the NCI-N87 cells in the H group compared to the control group (**; $P<0.01$) and in the H+R group compared to the H group (*; $P<0.05$). The results are the average of the three independent experiments.

Impact of Relaxin 2 treatment on hypoxia-associated oxidative damage

The administration of RLXH2 treatment resulted in a noteworthy augmentation of the activities of various antioxidant enzymes, as illustrated in Table 1. This outcome underscores the pivotal role played by RLXH2 in bolstering the cellular defence mechanisms against oxidative stress. In effect, the treatment with RLXH2 emerges as a safeguarding strategy for gastric cancer cells, as it triggers a robust upregulation in the expression levels of antioxidant enzymes.

Table 1: Assessment of antioxidant enzyme activities in the study groups

Antioxidant enzymes	Control*	Hypoxia*	Hypoxia+RLXH2*
SOD	80 ± 3.2	52 ± 2.7	67 ± 3.4
CAT	51 ± 2.5	19 ± 1.6	34 ± 1.9
GPX	31 ± 2.1	13 ± 1.2	25 ± 1.4

RLXH2; Relaxin 2, SOD; Superoxide dismutase, CAT; Catalase, GPX; Glutathione peroxidase, and *; U/mg protein.

Relaxin 2 increases nuclear factor erythroid 2-related factor 2 expression

We assessed the impact of RLXH2 on total Nrf2 levels in the gastric cancer cells. Western blot analysis was performed to determine Nrf2 expression before and after RLXH2 treatment by using specific antibodies against Nrf2 and a control (β-actin). RLXH2 increased expression of the Nrf2 protein compared to the control sample (Fig.2A). As shown in Figure 2B, there was an approximately six-fold increase in Nrf2 protein expression in the RLXH2 treated cells compared to the control (0 hour).

There was a significant increase in Nrf2 mRNA in the NCI-N87 cells after RLXH2 treatment compared to the control (Fig.2B).

Nuclear factor erythroid 2-related factor 2 translocation and HO-1 levels

Western blot analysis was carried out to determine nuclear fraction purity by using primary antibodies against GAPDH and the histone 4 proteins. The antibody against GAPDH did not show any band in the nuclear fraction; on the other hand, the antibody against histone 4 caused a clear band (Fig.3A).

The possible nuclear translocation of Nrf2 after RLXH2 treatment was assessed by Western blot. We cultured the NCI-N87 cells in 60 mm plates for 24 hours, and then treated them with RLXH2 (12 hours). Our analysis of Nrf2 expression indicated that the untreated nuclear fraction did not show any Nrf2 protein band, whereas there was a protein band for Nrf2 expression in the RLXH2 treated nuclear fraction at 12 and 24 hours (Fig.3B).

Inside the nucleus, Nrf2 can activate a wide range of genes and promote cell survival against oxidative stress. Enzymes activated by the Nrf2/ARE pathway include HO-1, GPX, CAT, SOD, GST, thioredoxin, and NQO-1. HO-1 is the main protein activated by Nrf2 (22). Therefore, we assessed the protein levels of HO-1 in the NCI-N87 cells. Figure 3C and D shows HO-1 time-dependent expression levels in the RLXH2-treated gastric carcinoma cells. HO-1 expression significantly increased in the RLXH2-activated gastric carcinoma cells.

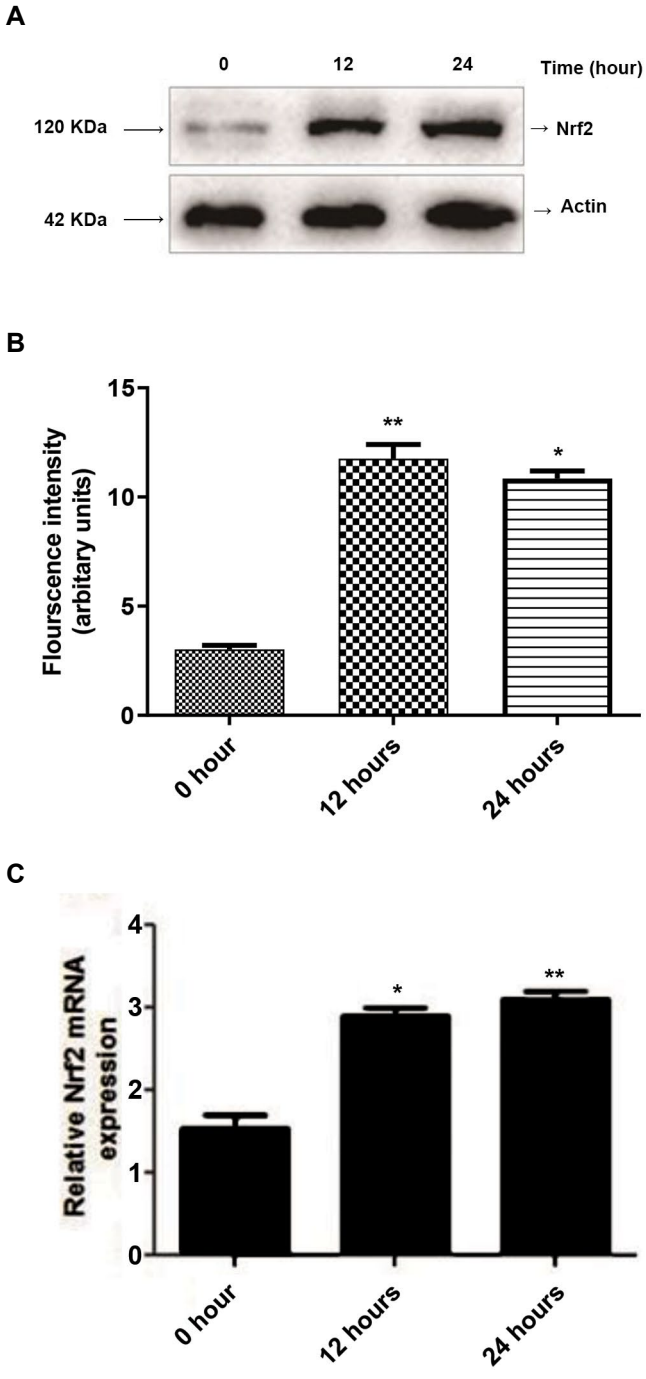


Fig.2: Nuclear factor erythroid 2-related factor 2 protein and mRNA levels. **A.** Nrf2 expression before and after relaxin 2 (RLXH2) treatment. **B.** Densitometry of Nrf2 bands (**; P<0.01 at 12 hours compared to 0 hours, and P<0.05 at 24 hours compared to 12 hours). **C.** Nrf2 mRNA levels significantly increased after RLXH2 treatment (*; P<0.05 both for 12 hours compared to 0 hour and for 24 hours compared to 12 hours). The results are the average of an independent experiment.

Impact of nuclear factor erythroid 2-related factor 2 silencing on relaxin 2 treatment

Figure 4A shows western blot of nuclear (N) and cytosolic (C) protein fractions. Nuclear fraction did not detect any GAPDH band; however a prominent protein band of Histone 4 was detected. As shown in Figure 4B (top panel), Nrf2 antibody detected a protein band in the control, while the Nrf2 protein was appreciably down-regulated in the siRNA transfected cells. We also assessed nuclear factor erythroid 2-related factor 2 (Nrf2) silencing on relaxin 2 (RLXH2) induced antioxidant enzymes in these NCI-N87 cells (Table 2). The results indicated that Nrf2 silencing partially blocked the RLXH2 induced antioxidant enzymes.

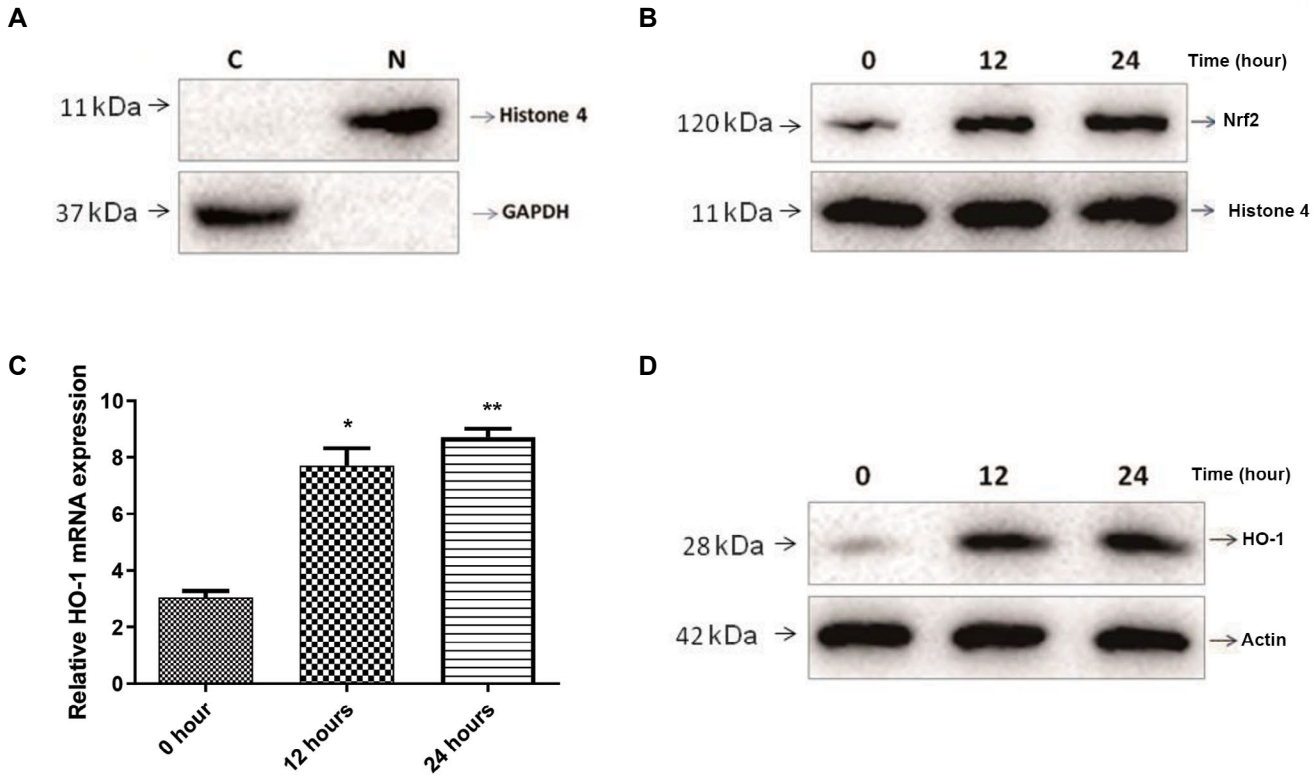


Fig.3: Nuclear factor erythroid 2-related factor 2 (Nrf2) translocation to the nucleus. **A.** Western blot assessment of nuclear (N) and cytosolic (C) protein fractions. The nuclear fraction did not detect any GAPDH band; however, there was a prominent protein band for histone 4. **B.** Western blot assessment of relaxin 2 (RLXH2) activated nuclear fraction. The untreated nuclear fraction did not show any band with the Nrf2 antibody; however, there was a protein band for Nrf2 in the RLXH2-activated nuclear fraction at 12 and 24 hours. **C.** There is increased expression of HO-1 mRNA in the RLXH2-activated NCI-N87 cells (12 hours and 24 hours) with $P<0.05$ at 12 hours compared to 0 hours, and $P<0.01$ at 24 hours compared to 12 hours). **D.** There is a significant increase in HO-1 expression level in the RLXH2-activated NCI-N87 cells (12 hours and 24 hours). The results are the average of an independent experiment. *; $P<0.05$ and **; $P<0.01$.

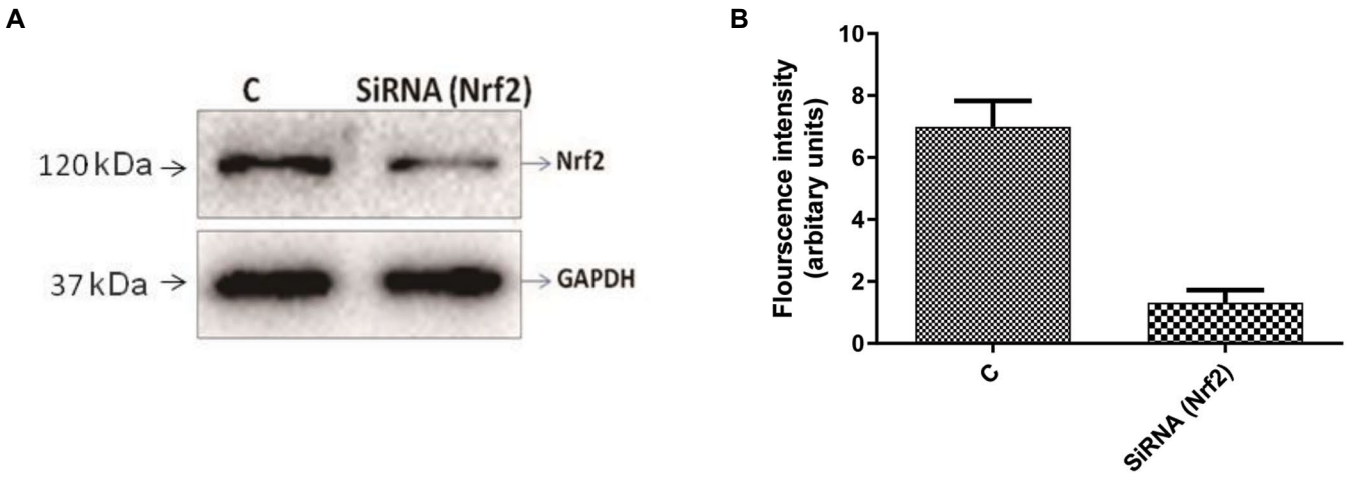


Fig.4: siRNA mediated down-regulation of nuclear factor erythroid 2-related factor 2 (Nrf2) in NCI-N87 cells. **A.** The cells were transfected with Nrf2-specific siRNA or with control siRNA (C). **B.** Immunoblotting was used to confirm down-regulation of Nrf2 with GAPDH as the control. The results are the average of an independent experiment.

Table 2: Impact of nuclear factor erythroid 2-related factor 2 silencing on relaxin 2 induced antioxidant enzymes in NCI-N87 cells

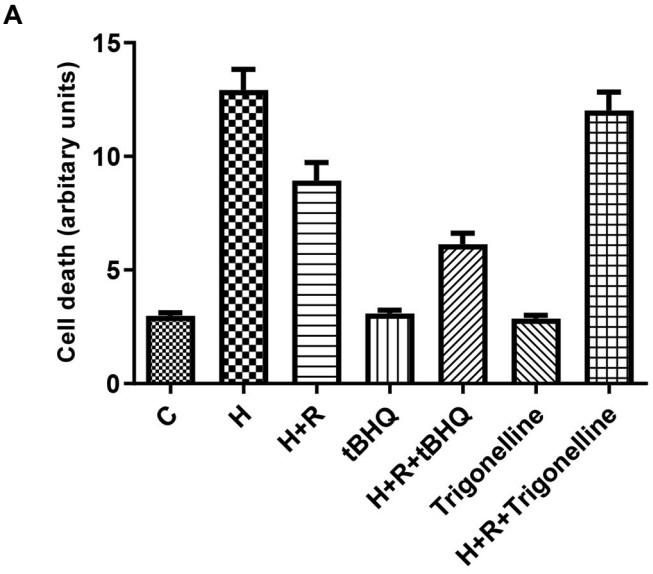
Antioxidant enzymes*	Control	Hypoxia	Hypoxia+RLXH2	Hypoxia+RLXH2+Nrf2-siRNA
SOD	78 ± 3.1	42 ± 2.3	51 ± 2.9	46 ± 1.9
CAT	55 ± 1.9	21 ± 1.6	32 ± 2.6	25 ± 2.1
GPX	30 ± 2.8	15 ± 1.3	24 ± 1.7	19 ± 2.0

SOD; Superoxide dismutase, GPX; Glutathione peroxidase, CAT; Catalase, *; Results are reported as U/mg protein.

Impact of relaxin 2 on hypoxia-associated cell apoptosis

We examined the impact of Nrf2 translocation cell death. NCI-N87 cells were cultured and incubated with agents that either inhibited or increased Nrf2 nuclear translocation. Figure 5A shows that the hypoxic condition significantly resulted in cell death. However, treatment of cells with RLXH2 (H+R) significantly reduced hypoxic-associated cell death compared to the hypoxia (H) group. In order to further prove the protective role of RLXH2 in NCI-N87 cells, we treated some of the cells with an Nrf2 inhibitor and others with tBHQ, an Nrf2 enhancer. Figure 5A shows that the Nrf2 inhibitor (trigonelline) eliminated RLXH2 associated cell protection, whereas treatment with the Nrf2 activator (tBHQ) increased cellular protection against hypoxia-associated cell death compared to RLXH2 treatment only.

HO-1 increased in the presence of RLXH2 in the NCI-N87 cells. Therefore, we sought to examine the role of HO-1 activation on hypoxia-associated cell death. The cells were cultured overnight and the next day, they were treated as RLXH2 only or RLXH2 with ZnPPIX. There was acute cell death in the NCI-N87 cells of the hypoxia group (H, Fig.5B). Treatment with RLXH2 (H+R) significantly reduced hypoxia-associated cell death. Inhibition of HO-1 expression significantly eliminated RLXH2 mediated cell protection.



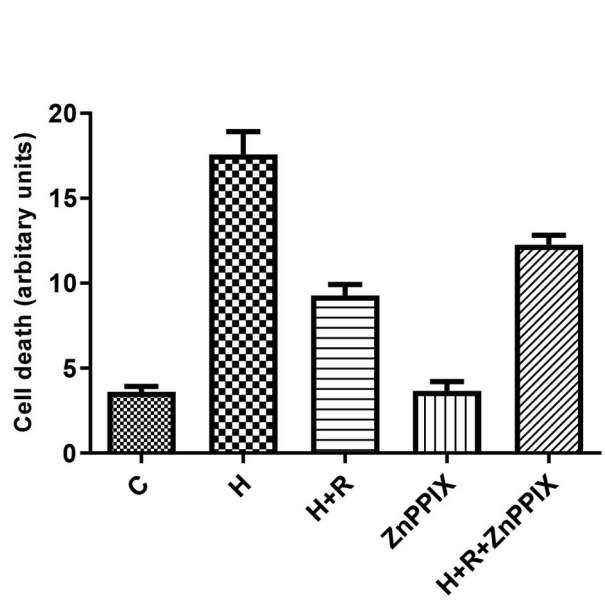


Fig.5: Hypoxia-associated cell apoptosis and role of nuclear factor erythroid 2-related factor 2 (Nrf2). **A.** Treatment with relaxin 2 (RLXH2) significantly reduces cell apoptosis. The protection offered by RLXH2 is eliminated when Nrf2 is inhibited and increases during activation of Nrf2. **B.** RLXH2 mediated cell protection and HO-1 inhibition. NCI-N87 cells cultured overnight are treated with RLXH2 only or RLXH2 with ZnPPIX. Treatment with ZnPPIX eliminates RLXH2 mediated cell protection. The results are the average of an independent experiment. C; Control group, H; Hypoxia group, and H+R; Hypoxia with RLXH2 treatment group.

Discussion

Hypoxia is common in different types of cancers and it causes activation of hypoxia-associated pathways in these cancer cells. Hypoxia within cells often results in oxidative stress, which can result in cellular damage and death. It has been reported that relaxin protect cells against hypoxia-associated cellular damage and death in different types of cancer cells (6, 23). Relaxin-associated cancer growth and invasion is a well-known phenomenon in thyroid, prostate, breast and other cancer models (24-26). In most, over-expression of relaxin has been observed, and this activates different protective signalling pathways (27-29). The role of relaxin in cancers is not fully understood but has recently emerged as a therapeutic target to counter the pro-cancer effects of enhanced relaxin levels (3). It has been reported in prostate cancer cells that down-regulation of relaxin decreases tumour formation in nude mice. LDH release by the cultured cells is a marker of cell death (11). Waza et al. (19) have reported

that RLXH2 successfully suppressed LDH release from cells with hypoxia. Here, we observed that treatment of RLXH2 prevented LDH release from hypoxia challenged gastric cancer cells.

Normally, cells remain safe from oxidative damage by the presence of various antioxidant enzymes along with GST, GSH, vitamin C, GPX, CAT, and SOD, among others (30). A proper balance of ROS is important for cells to function normally; however, the imbalance in ROS formation results in oxidative stress (31). Excessive production of ROS is linked with cellular damage, which is mainly due to enzyme inactivation, lipid peroxidation, and changes in nucleic acids (32). Hypoxia can cause excessive ROS production within cells (33) and oxidative damage to the cells, which eventually induces apoptosis or necrosis (34). Waza et al. (19) have reported that RLXH2 successfully suppressed hypoxia-associated ROS production and apoptosis. In the current study, treatment of NCI-N87 cells with RLXH2 (15 nmol/L) activated different antioxidant enzymes, and thereby decreased hypoxia-associated ROS formation in the NCI-N87 cells.

Nrf2 regulates the expression of different antioxidant enzymes (HO-1, GST, COX-2) (35, 36). It is a major defensive protein to combat oxidative stress in cancer cells (37). Nrf2 knockout cells are more prone to H_2O_2 induced cellular injury (38) and its over-expression within cells protects against injury from oxidative stress (14). Waza et al. (19) have reported that RLXH2 successfully activated the Nrf2/HO-1 signalling pathway. Since relaxin offered cellular protection against oxidative damage, we designed the current experiment to investigate the impact of RLXH2 on the Nrf2/HO-1 pathway (a major cellular defence against oxidative stress). We observed increased Nrf2 expression after RLXH2 treatment in the gastric cancer cells. Treatment with RLXH2 treatment enhanced nuclear translocation of Nrf2 in gastric cancer cells, and subsequently increased HO-1 levels. Furthermore, we observed that RLXH2 significantly eliminated hypoxia-induced apoptosis in these gastric cancer cells. The translocation of Nrf2 has been observed to undergo enhancement and inhibition upon exposure to tBHQ and trigonelline, respectively (39, 40). We found that incubation with tBHQ increased RLXH2 cell protection, while trigonelline abrogated this protection.

Conclusion

RLXH2 appears to be a promising therapeutic candidate for gastric cancer treatment because it offers protection against hypoxia-induced oxidative damage and cell death. Activation of the Nrf2/HO-1 pathway by RLXH2 provides a potential avenue for the development of targeted therapies that can enhance cellular antioxidant defences and counteract the detrimental effects of hypoxia. Further research and clinical investigations are necessary to fully exploit the therapeutic potential of RLXH2 and bring it closer to clinical application, and ultimately benefit patients with gastric cancer and other hypoxia-related disorders.

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Authors' Contributions

L.W.; Conception, design of the manuscript, and experimental work. Y.Z.; Data acquisition or data analysis and interpretation. H.L.; Experimental work and drafting of the manuscript. K.H.; Final approval of the manuscript and repeatation of experimental work. All authors read and approved the final manuscript.

References

1. Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocr Rev. 2004; 25(2): 205-234.
2. Wei X, Yang Y, Jiang YJ, Lei JM, Guo JW, Xiao H. Relaxin ameliorates high glucose-induced cardiomyocyte hypertrophy and apoptosis via the Notch1 pathway. Exp Ther Med. 2018; 15(1): 691-698.
3. Thanasupawat T, Glogowska A, Nivedita-Krishnan S, Wilson B, Klonisch T, Hombach-Klonisch S. Emerging roles for the relaxin/RXFP1 system in cancer therapy. Mol Cell Endocrinol. 2019; 487: 85-93.
4. Bani D, Pini A, Yue SK. Relaxin, insulin and diabetes: an intriguing connection. Curr Diabetes Rev. 2012; 8(5): 329-335.
5. Bruell S, Sethi A, Smith N, Scott DJ, Hossain MA, Wu QP, et al. Distinct activation modes of the relaxin family peptide receptor 2 in response to insulin-like peptide 3 and relaxin. Sci Rep. 2017; 7(1): 3294.
6. Radestock Y, Hoang-Vu C, Hombach-Klonisch S. Relaxin reduces xenograft tumour growth of human MDA-MB-231 breast cancer cells. Breast Cancer Res. 2008; 10(4): R71.
7. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer. 2002; 2(1): 38-47.
8. Pouysségur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature. 2006; 441(7092): 437-443.
9. Nordgren IK, Tavassoli A. Targeting tumour angiogenesis with small molecule inhibitors of hypoxia inducible factor. Chem Soc Rev. 2011; 40(8): 4307-4317.
10. Tarnawski A, Pai R, Deng X, Ahluwalia A, Khomenko T, Tanigawa T, et al. Aging gastropathy-novel mechanisms: hypoxia, up-regulation of multifunctional phosphatase PTEN, and proapoptotic factors. Gastroenterology. 2007; 133(6): 1938-1947.
11. Jing L, Li Q, He L, Sun W, Jia Z, Ma H. Protective effect of tempol against hypoxia-induced oxidative stress and apoptosis in H9c2 cells. Med Sci Monit Basic Res. 2017; 23: 159-165.
12. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med. 2000; 28(10): 1456-1462.
13. Granger DN, Kvietys PR. Reperfusion injury and reactive oxygen species: the evolution of a concept. Redox Biol. 2015; 6: 524-551.
14. Ma Q. Role of nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol. 2013; 53: 401-426.
15. Jung BJ, Yoo HS, Shin S, Park YJ, Jeon SM. Dysregulation of NRF2 in cancer: from molecular mechanisms to therapeutic opportunities. Biomol Ther (Seoul). 2018; 26(1): 57-68.
16. Pouremamali F, Pouremamali A, Dadashpour M, Soozangar N, Jeddi F. An update of Nrf2 activators and inhibitors in cancer prevention/promotion. Cell Commun Signal. 2022; 20(1): 100.
17. Kitamura H, Motohashi H. NRF2 addiction in cancer cells. Cancer Sci. 2018; 109(4): 900-911.
18. Zhang M, An C, Gao Y, Leak RK, Chen J, Zhang F. Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. Prog Neurobiol. 2013; 100: 30-47.
19. Waza AA, Hamid Z, Bhat SA, Shah NUD, Bhat M, Ganai B. Relaxin protects cardiomyocytes against hypoxia-induced damage in in-vitro conditions: Involvement of Nrf2/HO-1 signaling pathway. Life Sci. 2018; 213: 25-31.
20. Waza AA, Andrabi K, Hussain MU. Protein kinase C (PKC) mediated interaction between conexin43 (Cx43) and K(+)(ATP) channel subunit (Kir6.1) in cardiomyocyte mitochondria: Implications in cytoprotection against hypoxia induced cell apoptosis. Cell Signal.

2014; 26(9): 1909-1917.
21. Ahmad Waza A, Andrabi K, Ul Hussain M. Adenosine-triphosphate-sensitive K+ channel (Kir6.1): a novel phosphospecific interaction partner of connexin 43 (Cx43). Exp Cell Res. 2012; 318(20): 2559-2566.
22. Waza AA, Hamid Z, Ali S, Bhat SA, Bhat MA. A review on heme oxygenase-1 induction: is it a necessary evil. Inflamm Res. 2018; 67(7): 579-588.
23. Chen TY, Li X, Hung CH, Bahudhanapati H, Tan J, Kass DJ, et al. The relaxin family peptide receptor 1 (RXFP1): An emerging player in human health and disease. Mol Genet Genomic Med. 2020; 8(4): e1194.
24. Vinall RL, Mahaffey CM, Davis RR, Luo Z, Gandour-Edwards R, Ghosh PM, et al. Dual blockade of PKA and NF-κB inhibits H2 relaxin-mediated castrate-resistant growth of prostate cancer sublines and induces apoptosis. Horm Cancer. 2011; 2(4): 224-238.
25. Hombach-Klonisch S, Bialek J, Trojanowicz B, Weber E, Holzhausen HJ, Silvertown JD, et al. Relaxin enhances the oncogenic potential of human thyroid carcinoma cells. Am J Pathol. 2006; 169(2): 617-632.
26. Cao WH, Liu HM, Liu X, Li JG, Liang J, Liu M, et al. Relaxin enhances in-vitro invasiveness of breast cancer cell lines by upregulation of S100A4/MMPs signaling. Eur Rev Med Pharmacol Sci. 2013; 17(5): 609-617.
27. Hombach-Klonisch S, Buchmann J, Sarun S, Fischer B, Klonisch T. Relaxin-like factor (RLF) is differentially expressed in the normal and neoplastic human mammary gland. Cancer. 2000; 89(11): 2161-2168.
28. Bigazzi M, Brandi ML, Bani G, Sacchi TB. Relaxin influences the growth of MCF-7 breast cancer cells. Mitogenic and antimitogenic action depends on peptide concentration. Cancer. 1992; 70(3): 639-643.
29. Tashima LS, Mazoujian G, Bryant-Greenwood GD. Human relaxins in normal, benign and neoplastic breast tissue. J Mol Endocrinol. 1994; 12(3): 351-364.
30. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev. 2014; 94(2): 329-354.
31. Nita M, Grzybowski A. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. Oxid Med Cell Longev. 2016; 2016: 3164734.
32. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012; 24(5): 981-990.
33. Görlach A, Dimova EY, Petry A, Martínez-Ruiz A, Hernansanz-Agustín P, Rolo AP, et al. Reactive oxygen species, nutrition, hypoxia and diseases: Problems solved? Redox Biol. 2015; 6: 372-385.
34. Balderas-Villalobos J, Molina-Muñoz T, Mailloux-Salinas P, Bravo G, Carvajal K, Gómez-Viquez NL. Oxidative stress in cardiomyocytes contributes to decreased SERCA2a activity in rats with metabolic syndrome. Am J Physiol Heart Circ Physiol. 2013; 305(9): H1344-H1353.
35. Kim JH, Xu EY, Sacks DB, Lee J, Shu L, Xia B, et al. Identification and functional studies of a new Nrf2 partner IQGAP1: a critical role in the stability and transactivation of Nrf2. Antioxid Redox Signal. 2013; 19(2): 89-101.
36. Waza AA, Hamid Z, Ali S, Bhat SA, Bhat MA. A review on heme oxygenase-1 induction: is it a necessary evil. Inflamm Res. 2018; 67(7): 579-588.
37. Vomund S, Schäfer A, Parnham MJ, Brüne B, von Knethen A. Nrf2, the Master regulator of anti-oxidative responses. Int J Mol Sci. 2017; 18(12): 2772.
38. Zhu H, Itoh K, Yamamoto M, Zweier JL, Li Y. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett. 2005; 579(14): 3029-3036.
39. Zagorski JW, Turley AE, Dover HE, VanDenBerg KR, Compton JR, Rockwell CE. The Nrf2 activator, tBHQ, differentially affects early events following stimulation of Jurkat cells. Toxicol Sci. 2013; 136(1): 63-71.
40. Arlt A, Sebens S, Krebs S, Geismann C, Grossmann M, Kruse ML, et al. Inhibition of the Nrf2 transcription factor by the alkaloid trigonelline renders pancreatic cancer cells more susceptible to apoptosis through decreased proteasomal gene expression and proteasome activity. Oncogene. 2013; 32(40): 4825-4835.