Biomedical Current Insights
DOI: https://doi.org/10.63626/ppvkb747
ISSN Online: XXXX-XXXX
ISSN Print: XXXX-XXXX

Research Article

Effects of a ribosome inactivating plant protein (riproximin) on transcriptomic profile of apoptosis pathway genes in breast cancer cells

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Abstract
Background: Treatment options for breast cancer are limited and the available possibilities are largely palliative in nature. In this context, searching for more effective therapeutic compounds is inevitable. Riproximin, a plant derived ribosomal inactivating protein, is one such kind of anticancer compound and has shown substantial anticancer effects while altering the major components of different molecular pathways including cell cycle, autophagy, cell stress and apoptosis. The purpose of this study was to investigate the effects of riproximin exposure on expressional levels of genes associated with apoptotic pathway in breast cancer cell lines.
Methods: In first phase, breast cancer cell lines (MDA-MB-231 and MCF-7) were exposed to riproximin, and toxic effects were identified by MTT dye reduction assay. Afterwards, the cell lines were exposed to riproximin in a separate experiment and expressional modulations of multiple genes were evaluated via real-time PCRs. Fold changes in this study were calculated by Livak method ($2^{\Delta\Delta Ct}$) while using untreated cells as controls.
Results: Proliferation (MTT) assay showed that riproximin induced promising cytotoxic effects in breast cancer cell lines. MCF-7 cells were more responsive towards riproximin exposure as compared to MDA-MB-231 cells. Expressional assessment of the selected genes confirmed the potential of riproximin to alter the levels in breast cancer cells. Specifically, in response to riproximin exposure, three most effectively induced genes in MDA-MB-231 cells were BIK (83fold), TNFSF9 (59fold) and NFKB1 (20fold). In MCF-7 cells, three most up-regulated genes after riproximin treatment were TNF (129fold), LTA (58fold) and TNFSF9 (17fold).
Conclusion: Riproximin bears significant cytotoxic potential against breast cancer cell lines. Expressional modulations in multiple apoptotic related genes are imposed by riproximin in the breast cancer cells. Further in *vitro* and in *vivo* studies are required to support the evaluation of riproximin in clinical settings against breast cancer cells, while targeting the apoptotic routes for cancer treatments.
Key Words: Breast cancer, Apoptosis, Riproximin, Protein, Ribosome inactivating

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INTRODUCTION

Breast cancer is the leading cause of cancer related mortalities worldwide. The most common type of breast cancer is ductal carcinoma. Other breast cancer types include ductal carcinoma in situ, invasive breast cancer and inflammatory breast cancer [1]. Histological type, tumour grade, and tumour stage are key parameters in diagnosis of breast cancer by using criteria as outlined in Nottingham Grading System. Treatment strategy is administered according to the subtype and stage of disease. There are different treatment strategies being used for breast cancer treatment which include chemotherapy, radiation therapy, hormonal therapy and surgery. Treatment options are limited for advanced stages of this cancer and the available opportunities are largely palliative in nature. In this context, searching for more effective therapeutic compounds is inevitable [2, 3].

Plants have been an attractive source of natural therapeutic compounds against various diseases including cancers. Plant-derived compounds can differentiate between cell types, being non-toxic to normal cells [4]. Almost a decade ago, a ~60 kDa active protein component was extracted and purified from the kernels of *Ximenia americana*, and was named as riproximin, a type two ribosome inactivating protein [5]. Ribosomes inactivating proteins (RIPs) are the plant proteins that stop protein synthesis by enzymatically damaging the ribosomes [6]. RIPs are

widely distributed in nature and originate from plants. In addition, other resources include microorganisms, algae, mushrooms and even insects. Based upon physical properties, RIPs are classified into three main groups i.e., type 1, 2 and 3. Different types of RIPs have been studied, and these proteins negatively affect the growth of tumour cells *in vivo* and *in vitro* [7-9].

Riproximin belongs to type 2 RIP with two polypeptide chains A and B. A-chain has N-glycosidase activity while B-chain has lectin domain for binding purposes. The mechanism of working of riproximin is through depurination of 28s RNA of eukaryotic ribosome. This depurination of 28s rRNA results in transcriptional arrest. Additional mechanisms for riproximin mediated signaling pathways that lead to apoptosis include ribotoxic response, stress induced mitochondrial pathway, down regulation of anti-apoptotic factors, NAD+ depletion of PARP hyper-activation and DNA damage due to nuclease activity. Riproximin is used to trigger apoptosis as cancerous cells evade apoptosis via various mechanisms. Triggering apoptosis is an alternative to chemotherapy which has toxic effects on both cancerous and normal cells while riproximin is non-toxic to healthy cells [10].

Anticancer effects of this protein have been evaluated against a variety of cancer cell lines including primary and metastatic cancer cells. Over the years, a series of experiments showed significant antineoplastic potential of riproximin in *in-vitro* studies. Recently, the antineoplastic effects of riproximin in breast and colorectal cancer cell lines have been evaluated by various functional assays. The results highlighted the significant inhibition of proliferation, migration, colony formation, induction of apoptosis and S-phase cell cycle arrest in selected cell lines. At molecular levels, riproximin modulates multiple signaling cascades leading to cytostatic and apoptotic effects in human breast and colorectal cancer cells [11, 12]. Avoiding the apoptotic routes is a hallmark of cancer cells and targeting such sensitive aspects via natural compounds could be a highly effective anticancer approach. Keeping in mind the apoptosis inducing ability, the current study was conducted to reveal the effects of riproximin on expressional modifications of apoptotic related multiple genes in breast cancer cells. Furthermore, the expressional modulations were used to design signaling cascades being affected by riproximin in the target breast cancer cells to induce death mechanisms.

METHODS

Cell Culture

Two Human breast cancer cell lines (MDA-MB-231 and MCF-7) were maintained in cell culture medium RPMI-1640, supplemented with 10% of FBS, 2mM L-glutamine, 100µg/ml of streptomycin, 100IU/ml of penicillin with standard humidification conditions of 5% CO2 and temperature of 37^0C. Routine culturing of cells was done (cells passaging 2-3 times/week) to maintain logarithmically growing cell population.

MTT Dye Reduction Assay

Cultured cells were treated with serial dilution of riproximin at pre-optimized cell densities (4000cells/well) in 96-well plates and exposed to various concentrations of riproximin (0.05-50ng/ml) for three-time intervals i.e., 24, 48 and 72 hours and toxic effects of riproximin against breast cancer cells were studied by a calorimetric MTT dye reduction assay. After the treatment intervals, MTT solution (10mg/ml in PBS) was added to the wells followed by incubation at standard conditions for 3 hours. Formazan crystals, formed by the viable cells, were dissolved by addition of 50µl of DMSO in each well. Optical densities of the samples were measured by using an ELISA reader at 540 nm and 645 nm reference filter. Cell survival rates were calculated as percentage of the untreated cells while inhibitory concentrations (ICs) were calculated by GraphPad Prism 9 software.

Treatment with Riproximin

For expressional profiling, cells were cultured in 6-well plates at a density of 150,000 cells/well/ 2ml of medium and next day, treated with three different concentrations of riproximin (1-50ng/ml) for 48 hours. Afterwards, the cell palettes were collected by trypsinizing the cells with 0.05% solution of trypsin-EDTA and collected by centrifugation at 1500-2000rpm for a duration of 5min followed by storage at -80^0C. Likewise, the cell palettes of untreated cells as controls were also collected and stored in similar conditions. These two cell lines were comprised of eight samples in total i.e., two controls (untreated MDA-MB-231 and MCF-7 cells) and six riproximin treated samples of MDA-MB-231 (5, 10, and 50ng/ml) and MCF-7 cells (1, 5, and 20ng/ml)

RNA Extraction and Quantification

Following the exposure period of cultured cells with riproximin total RNA was extracted (Thermo Fisher Scientific, Cat#K0731) from control and treated cell lines and was quantified by placing 2µl of extracted RNA on Nanodrop ND2000 designated area and readings were taken as prescribed protocol.

cDNA Synthesis and Verification

A total of 1500ng of extracted RNA, was used to synthesize the cDNA (40µl) by using Reverse Transcriptase, OligodT and dNTPs following the protocol of selected kit (Thermo Fisher Scientific, Cat#K1622). For verification purposes, a PCR based amplification of a reference gene (GAPDH) was done of prepared cDNA samples and amplified products were loaded on 2.5% agarose gel and visualized by gel electrophoresis.

Primer Designing and Optimization

A total of ten genes related to apoptosis pathway were selected and primers were designed by choosing gene specific sequences from NCBI Gene bank and using Primer 3 plus (Table 1) and optimized via gradient PCR methodology. After some consistent efforts, primers were optimized for their respective appropriate master mix requirements and cyclic conditions.

Real Time PCR and Data Analysis

By using cDNA generated from cell lines (MDA-MB-231 and MCF-7) untreated and treated with different concentrations of riproximin, qRT-PCR was performed by using SybrGreen fluorescence dye for selected optimized 10 genes. After normalization of data sets by using reference gene, the data was analyzed by calculating fold changes using Livak 2-ΔΔCT method by comparing Cq (quantification cycle) values of experimental (riproximin treated) and untreated control samples.

Table 1: Primer sequence for amplification of selected genes

Gene	Primer Sequence (F)	Primer Sequence (R)
BAK	AGAGTTCCAGACCATGTTGC	CATGCTGGTAGACGTGTAGG
BCL10	ACTGAAGTGAAGAAGGACGCC	CAACAAGGGTGTCCAGACCT
BIK	GTCCTGGGTGTCCTGCGAA	AAGATAACAGCAGCAGGCCG
FASLG	CTGGGGATGTTTCAGCTCTTC	CTTCACTCCAGAAAGCAGGAC
GAD45A	AACGGTGATGGCATCTGAAT	CCCTTGGCATCAGTTTCTGT
LTA	CTCACCTCATTGGAGACCCC	CCACCTGGGAGTAGACGAAG
NFKB1	CCTACGATGGAACCACACCC	ATCTGCTCCTGCTGCTTTGA
TNF	CCTGCTGCACTTTGGAGTGA	GAGGGTTTGCTACAACATGGG
TNFSF9	CTTCCTCACGCTCCGTTTCT	ACTGGTCTCATAAATGGTTGTTTGA
TRAF3	GTCCTGACAGAAGAGAACTCC	TTTAGCGGCGGGTTAGTCTG

RESULTS

Toxicity of Riproximin

Toxic effects of riproximin against breast cancer cell lines MDA-MB-231 and MCF-7 were studied by MTT dye reduction assay and ICs were identified with the help of GraphPad Prism 9 software. Among the two cell lines, metastatic MDA-MB-231 cells were less responsive as shown by higher IC50 especially after 48 hours of exposure time. However, the differences between the sensitivity of the two cell lines were reduced after longer exposure (72 hours) with riproximin. IC50 values for selected breast cancer cell lines MDA-MB-231 and MCF-7 are given in Table 2.

RNA Extraction and cDNA Synthesis

RNA was extracted from untreated control and treated breast cancer cell lines (MDA-MB-231 and MCF-7) and measured by nanodrop technology. Extraction procedure was successful as a good quantity (55-357ng/µl/sample) and quality (260/280 ratio: 1.9-2.0) of RNA was extracted from the samples. Following the protocol of selected reverse transcriptase enzyme kit (Thermo Fisher Scientific, Cat# K1622), cDNA was synthesized by using extracted total RNA and verified by PCR based amplification of a reference gene (results not shown here).

Expressional Analysis of Genes

Effect of riproximin on the selected genes (BIK, BAK1, BCL10, FASLG, GAD45A, LTA, NFKB1, TNF, TNFSF9 and TRAF3) in breast cancer cell lines (MDA-MB-231, and MCF-7) were investigated after exposure with three different concentrations of riproximin. The modifications in four (LTA, TNF, TNFSF9, TRAF3) were concentration dependent on both cell lines, which reflected continuous induction in response to higher concentrations of riproximin exposure. NFKB1 and BIK show almost the same trend as highest alterations were reflected in response to the highest riproximin concentration applied.

In addition to this, there were some cell specific responses as well as shown by concentration dependent induction of BAK1 was observed for MDA-MB-231 cells, while MCF-7 cells did not follow this pattern. In contrast, BCL10 and FAS genes were highly induced against the lowest concentrations of riproximin (1ng/ml) in MCF-7 cells, while the effects were minimized at highest concentrations (20ng/ml). Overall modifications in the genes are shown in Figures 1.

Table 2: IC50 concentrations (ng/ml) after 48 and 72 hours of treatment.

	Breast Cancer Cell Lines	
	MDA-MB-231	**MCF-7**
48 h	10.2ng/ml	1.8ng/ml
72 h	2.9ng/ml	1.2ng/ml

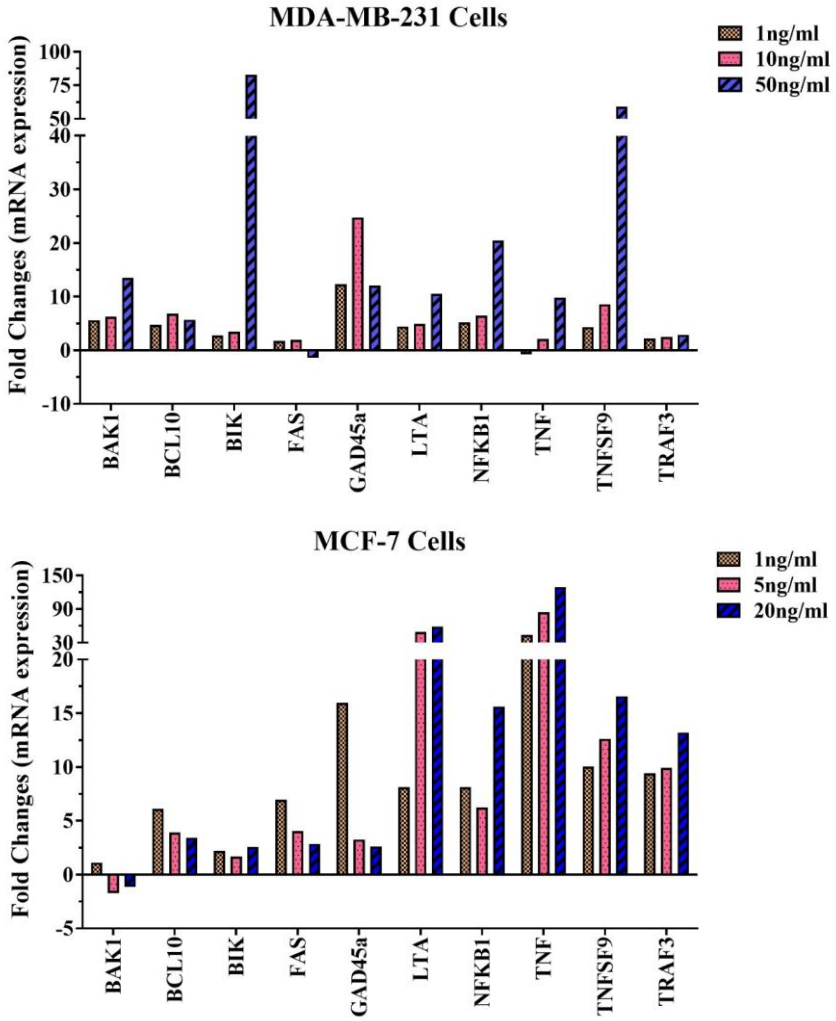

Figure 1: Fold changes of selected genes in MDA-MB-231 and MCF-7 cells. The cells were exposed to riproximin, and expressional changes were determined by using real-time PCRs and Livak method.

DISCUSSION

Cancer is a dreadful disease, causing a great deal of mortality and morbidity throughout the world. There are more than 100 types of cancers known and breast cancer is the second leading malignancy among them. Management of breast cancer is challenging as treatment strategies are not sufficient to control the burden of breast cancer and available treatments are costly and impose huge side effects as well. For this reason, focus should be shifted towards novel naturally occurring therapeutic agents to control the side effects and plants are being used for centuries against various diseases including cancers. Among these seeds of *X. americana* have been used by the local inhalers for treatment of cancers in African countries [12]. Active fractions of these seeds named riproximin have been tested against various cancer cell lines and animal models for their anti-neoplastic effects [11-14]. The purpose of this study was to evaluate the effects of riproximin on apoptosis related genes in breast cancer primary (MCF-7) and metastatic (MDA-MB-231) cell lines.

Initially, MTT dye reduction assay was used to determine and confirm the sensitivity of breast cancer cell lines towards riproximin by exposing the cell lines to increasing concentrations of riproximin (up to 50ng/ml) for 24-72 hours. According to the results, primary cell lines MCF-7 were more responsive as compared to metastatic MDA-MB-231 cell lines towards riproximin which is in line with already available literature [11]. This is indicated by IC50 values at 48 and 72 hours of exposure periods which are 1.8 and 1.2ng/ml for MCF-7 and of 10.2 and 2.9ng/ml for MDA-MB-231. This difference in sensitivity may be due to certain factors like affinity difference of riproximin towards both cell lines or the molecular difference between the two breast cancer cell lines (ER/PR +ve/-ve or presence of wild or mutated type P53).

To determine the effect of riproximin on apoptosis related genes in two breast cancer cell lines, the cells were exposed to different concentrations of riproximin (MDA-MB-231: 1, 10 and 50ng/ml, MCF-7: 1, 5 and 20ng/ml) for 48 hours. After the exposure period, RNA was extracted from treated cell lines, quantified and converted to cDNA for subsequent real time amplification by using gene specific primers. Results of these expression profiling experiments showed comparable outcomes which in turn demonstrate that riproximin has effective potential to induce apoptosis related genes in primary and metastatic breast cancer cells. However, the important point is that the level and type of apoptosis related genes induced by riproximin in these cells varied substantially. For example, BIK and GADD45A were one the most effectively induced genes in MDA-MB-231 cells. Among these, BIK is a BCL-2 interacting killer pro-apoptotic protein and plays an important role in initiating apoptosis [15]. This, in turn shows that riproximin has potential to interact with intrinsic arm of apoptosis and can induce vital mitochondrial associated gene families. GADD45A is major indicator of cell stress and leads the way to induce apoptosis by fragmentation of DNA and nuclei [16]. Current data shows that riproximin can act like a cell stress inducer and can up-regulate the related gene families like GADD. In MCF-7 cells, LTA and TNF were the most effectively induced genes in response to riproximin exposure. LTA and TNF have substantial importance in survival and death mechanisms. Induction of these genes shows that riproximin has potential to alter the expression of master regulators to induce apoptotic process in breast cancer cells. In addition to above mentioned genes, NFKB1, a very important transcription factor was shown to be upregulated effectively in both breast cancer cell lines. This transcription factor has tremendous importance for regulating the release of cytokines, immune responses and stress mechanisms. Further studies should also be aimed at investigating the induction of NFKB1 via riproximin in breast cancer cells as it could be a vital arm for inducing anticancer effects clinically. In a nutshell, riproximin bears significant cytotoxic potential against breast cancer cell lines. Expressional modulations in multiple apoptotic related genes are imposed by riproximin in the breast cancer cells. All in all, data reflected that riproximin is a promising antineoplastic compound with substantial potential to alter genetic expression and needs due attention for its considerations as anticancer agent.

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Ethics Approval: Not Applicable

Author Contributions: KU executed experiments and wrote the manuscript. AK helped with data analysis and assisted in experiments. AP supervised manuscript draft.

Competing Interests: None

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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