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Research Article

Antitumor potential of plant protein (Riproximin) against breast and colorectal cancer: Facts from functional and molecular investigations

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ABSTRACT

Background: Ribosomal inactivating proteins comprise a big domain and inactivate the ribosomal machinery of the target cells irreversibly and induce toxic effects. Riproximin is a type II ribosomal inactivating protein which has been found lethal against various cancer cells originating from different tissues including breast and colorectal. The purpose of this study was to evaluate the effects of riproximin exposure on cell cycle relevant genes in breast and colorectal cancer cells.
Methods: Toxic effects of riproximin in breast (MDA-MB-231, MCF-7) and colorectal cancer cell lines (SW480, SW620) were identified by MTT assay. AT molecular levels, the cell lines were treated with riproximin separately and expressional levels of the three S-phase cell cycle related genes (CDC6, MCM3, MCM5) were evaluated to identify the effects of riproximin. Untreated samples were used as controls throughout the study.
Results: Riproximin induced toxic effects in breast and colorectal cancer cells (IC_{50}<1.2ng/ml, 24 hours). Exposure of the cells with different concentrations of riproximin showed concentration dependent effects on expressional profile of the selected S-phase related genes. The selected genes were continuously down regulated by the protein in the breast and colorectal cancer cell lines. The riproximin associated effects were statistically significant.
Conclusion: Riproximin imposed significant cytotoxic and gene modulation effects in breast and colorectal cancer cells. Further studies are required to understand the molecular features affected by riproximin in cancer cells.
Key Words: Plants, Protein, Riproximin, Cancers, Cell death, Cell cycle, Genes

INTRODUCTION

Ribosomal inactivating proteins (RIPs) are plant originated proteins which can inactivate the ribosomal machinery and inhibit the translation irreversibly to induce toxic effects in target cells [1]. RIPs have been primary candidates for the toxic moiety of immunotherapeutic because of their selective toxicity. RIPs can be used as anti-cancerous drug. Ricin and viscum lectin are the well-known examples of RIPs in this context [2]. RIPs are classified into three classes: Type 1 RIPs; these are monomeric enzymes and share highly conserved active residues and secondary structure within the active site region. Some of the examples that fall in the category of Type 1 RIPs are pokeweed antiviral protein (PAP), saporin (from soapwort) and barley.

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Type II RIPs: these are highly toxic heterodimeric protein with the properties of enzymatic and lectin in polypeptide chains. Type II RIPs activity is due to A-chain that is linked with the galactose binding lectin i.e., B-chain through the di-sulfide bond. Ricin and Abrin are the examples of Type II RIPs [3]. Type III RIPs: these are synthesized as inactive precursors and further required proteolytic activity to be functional. RIPs have well-known anti-viral activity and play an important role in plant defense mechanisms. The anti-viral mechanisms by RIPs have not been resolved clearly. Anti-viral activity is not only present in plants, but it can also be seen in the cells of animals for both RNA and DNA viruses. RIPs do have anti-fungal activity. Three RIPs have been reported with antifungal activity and ability to inactivate fungal ribosomes i.e., Ricin-A Chain, saporin S-6 and an RIP from *Mirabilis expansa.* Several RIPs are presently being used in clinical trials for the treatment of cancer. Abrin and ricin are the two most potent toxic plant lectins. These RIPs can reduce the number of tumor cells and increase the effectiveness of the anti-tumor agents against various malignancies including

cervical carcinoma, leiomyosarcoma, fibrosarcoma, adenocarcinoma of the bladder, urethral vulvar, ovarian cancer and many others [4-6].

Riproximin is a type II RIP, comprised of heterodimer having two polypeptide chains (A and B-chains), which are linked together by intra-molecular di-sulfide bridge. A-chain of riproximin is responsible for the catalytic activity, while B-chain directs the binding to target cell surface molecules [7]. Riproximin showed significant antineoplastic potential against different cancer cell lines. Cytotoxicity of riproximin was investigated against leukemia, prostrate, pancreatic, colorectal and breast cancer. Riproximin imposed significant anticancer effects in these cancer cell lines and *in vivo* models for colorectal and pancreatic cancer liver metastasis [8, 9]. Activity of riproximin was investigated in 17 human and rat cells of pancreatic ductal adenocarcinoma (PDAC). Cytotoxic effects were determined by MTT assay. Riproximin showed high but varied cytotoxicity in PDAC cells [8]. Riproximin induced significant cytotoxic effects in breast cancer cells (MDA-MB-231 and MCF-7). Furthermore, significant inhibition of migration and colony formation was observed in the cells. Riproximin also induces S phase cell cycle arrest and apoptosis in the target cancer cells [10, 11]. Riproximin showed significant cytotoxic and cytostatic effects in colorectal cancer cell lines. Inhibition of clonogenicity and migration was also observed after the exposure of riproximin. Furthermore, riproximin induced high expression levels of growth arrest and DNA damage (GADD family) and anticancer genes (IL24) in colorectal cancer cells [10]. In this study, focus was to highlight the effects of riproximin exposure on cell cycle related machinery, where deregulation of S-phase related genes was investigated after the exposure with protein in primary and metastatic breast and colorectal cancer cells.

METHODS

Cell Culture

Two human breast cancer cell lines i.e., MDA-MB-231 and MCF-7 and two colon adenocarcinoma cancer cell lines i.e., SW480 and SW620 purchased from ATCC were cultured in RPMI-1640 medium supplemented with fetal bovine serum (10%), 2mM L-glutamate, streptomycin (100µg/ml) and penicillin (100IU/ml).

Cell Proliferation

MTT dye reduction assay was performed to observe cell proliferation (viability) following the treatment with riproximin. The cell lines were cultured (4000cells/well) in 96-well plates and exposed to various concentrations of riproximin (0.05-40ng/ml) for three-time points i.e., 24, 48 and 72 hours. After the treatment intervals, MTT solution 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 (540 nm filter).Cell survival rates were calculated as percentage of the untreated cells while inhibitory concentrations (ICs) were calculated by GraphPad Prism software.

Expression Profiling

Total RNA extraction was carried out using RNeasy Mini Kit (Qiagen) according to manufacturer's protocol and quantified using spectrophotometric system, Nanodrop ND 2000. One microgram of extracted total RNA was used to synthesize cDNA (20µl) by using Maxima Reverse Transcriptase (Thermo Scientific) for each sample. A PCR reaction was set-up to check and validate cDNA synthesis by amplifying a reference gene (GAPDH) and gel electrophoresis. Genes associated with the S-phase of cell cycle were selected (CDC6, MCM3 and MCM5). Primers were designed by choosing gene sequence of the selected genes from NCBI Gene bank and using Primer3 software (Table 1).

Real Time PCRs

qRT-PCR was performed by using SybrGreen florescence dye for the selected 3 genes and cDNA samples from four cell lines (MDA-MB- 231, MCF-7, SW480, SW620) treated with three concentrations of riproximin (IC_{25}, IC_{50} or IC_{75}). ΔΔCT method was used to find out expressional changes while comparing the riproximin treated samples with untreated controls. β-actin was used as a reference gene in these amplifications' procedure. Data sets from treated experimental and untreated control groups were used to analyze expressional modifications (fold changes) of selected genes by student *t-test*. *P* value less than 0.05 was considered as statistically significant.

Table 1: Sequence of primers of genes

Gene	Primer Sequence (F.)	Primer Sequence (R.)
CDC6	CTTAAGCCGGATTCTGCAAG	ATACATCCTGGCCTTTGCTG
MCM3	TTCCTCAGCTGTGTGGTCTG	CTCCTGGATGGTGATGGTCT
MCM5	CATCCGAAGCTCCTACATCC	TGTCTCCTCGGCGAGTAAGT
β-Actin	TCCACCTTCCAGCAGATGTG	GCATTTGCGGTGGACGAT
GAPDH	ACGGATTTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT

RESULTS

Riproximin Toxicity

Toxic effects of riproximin against breast and colorectal cancer cells were studied by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide) dye reduction assay as described above. Inhibitory concentrations (ICs) were identified by the help of GraphPad Prism 6 software, while IC_{50} values for selected cancer cells are given in Table 2. Riproximin showed comparable toxic effects in the cancer cells lines. Overall, when compared the 72 hours data, MCF-7 cells were more sensitive towards riproximin followed by SW620, SW4280 and MDA-MB-231 cells.

Verification of cDNA synthesis

To verify the cDNA synthesis process, a reference gene (GAPDH) was amplified by conventional PCR methodology. Afterwards, the amplified product was visualized by agarose gel electrophoresis (Figure 1, 2). Discrete bands of amplified products showed a good quality synthesized cDNA from the samples.

Expressional Profiling of Genes

Effect of riproximin on three S-phase related genes (CDC6, MCM3, MCM5) in breast (MDA-MB-231, MCF-7) and colorectal cancer cells (SW480, SW620) were investigated after exposure of the four cell lines with the test compound (IC_{25}, IC_{50}, IC_{75}) for 48 hours. Primers were designed for the three mentioned genes and optimized by amplifying two cDNA samples (untreated SW480 and SW620) by conventional PCR methodology (results not shown). Optimized primers were used to identify expressional changes via qRT-PCRs in subsequent amplification procedures. In a concentration dependent format, the three selected genes were down-regulated in breast cancer cell lines except for slight induction in MDA-MB-231 cells at lower concentration (IC_{25}) of riproximin (Figure 3). In colorectal cancer cells, the expression of the three genes was inhibited in SW480 cells at highest concentrations of riproximin (IC_{75}), while in SW620 cells the three genes were downregulated in concentration defendant format (Figure 4).

Table 2: IC_{50} concentrations after 24, 48 and 72 hours of riproximin treatment

	Breast Cancer Cells		Colorectal Cancer Cells	
	MDA-MB-231	**MCF-7**	**SW480**	**SW620**
24 hours	1.2 ng/ml	0.15 ng/ml	1.1 ng/ml	0.75 ng/ml
48 hours	3.6 ng/ml	0.42 ng/ml	2.1 ng/ml	1.5 ng/ml
72 hours	10.1 ng/ml	1.1 ng/ml	4.3 ng/ml	3.1 ng/ml

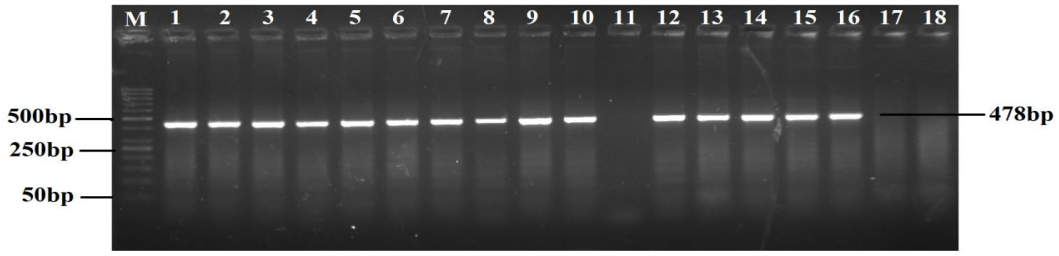

Figure 2: Electrophoresed cDNA of MDA-MB-231 and MCF-7: Sample distribution (left to right): DNA marker (50bp) Well 1-8: MDA-MB-231 (Untreated Control, IC_{25}, IC_{50}, and IC_{75} (each sample is in duplicate) Well 9-16: MCF-7 (Untreated Control, IC_{25}, IC_{50}, and IC_{75} (each sample is in duplicate) Well 17-18: RT –ve and PCR –ve controls.

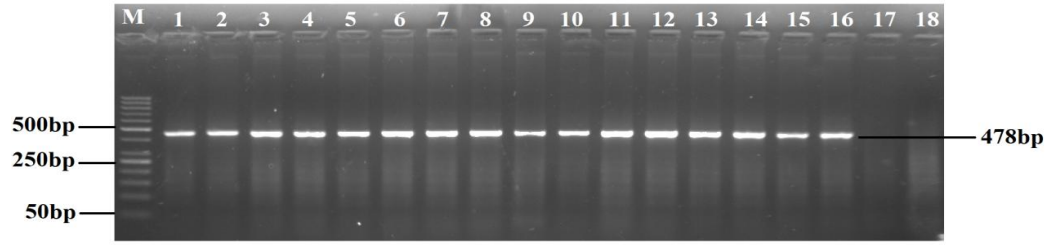

Figure 1: Electrophoresed cDNA of SW480 and SW620: Sample distribution (left to right): DNA marker (50bp) Well 1-8: SW480 (Untreated Control, IC_{25}, IC_{50}, and IC_{75} (each sample is in duplicate) Well 9-16: SW620 (Untreated Control, IC_{25}, IC_{50}, and IC_{75} (each sample is in duplicate) Well 17-18: RT –ve and PCR –ve controls.

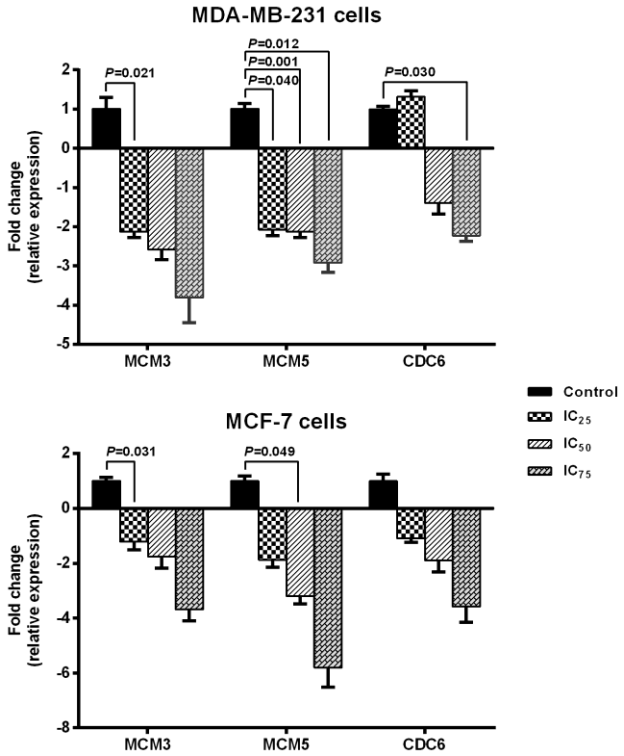

Figure 3: Expressional changes in S phase related genes in breast cancer cells treated with different inhibitory concentrations of riproximin. The selected S phase related three genes were inhibited in a concentration dependent format by riproximin in breast cancer cells.

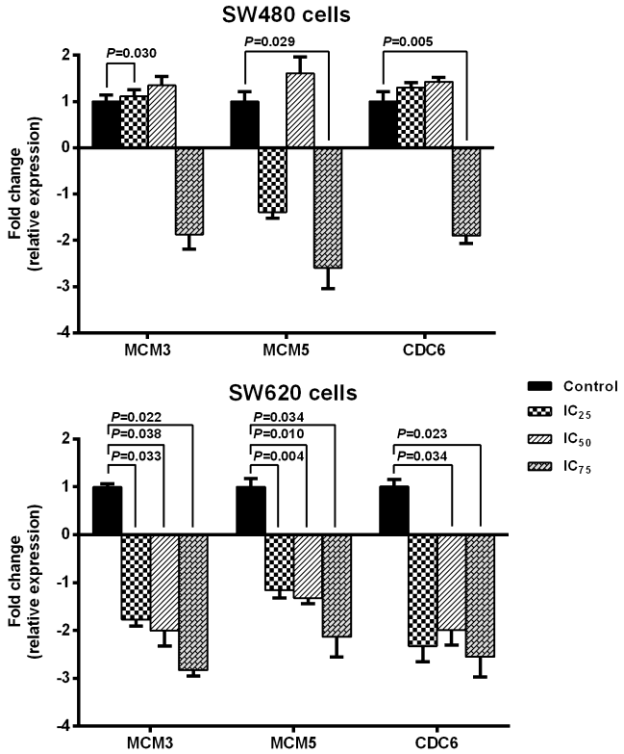

Figure 4: Expressional changes in S phase related genes in colorectal cancer cells treated with different inhibitory concentrations of riproximin. The selected S phase related three genes were inhibited in a concentration dependent format by riproximin in SW620 cells, while there were differential effects of the test compound on SW480 cells.

DISCUSSION

Breast cancer and colorectal cancer are two leading malignancies and account for more than 20% of cancer related mortalities worldwide [12]. Advanced stages (metastatic phases) of these cancers are difficult to treat and cause significant morbidity and mortality. The scenario shows that various chemotherapeutic and targeted agents, being used to treat these cancers, are insufficient to control the disease burden. In addition, synthetic anticancer agents cause several side effects. In the present situation, it is inevitable to look for naturally occurring anticancer compounds with better efficacy and minimal side-effects. In this context, plants comprise big genera in the world and produce numerous therapeutic entities naturally. RIPs encompass one such group of naturally occurring anticancer compounds [13]. Different members of RIPs are being used clinically to treat cancers. Riproximin is relatively less known member of RIPs, which has shown tremendous potential as anticancer compound against various cancers *in vitro* and *in vivo* [8]. A variety of mechanistic ways have been proposed and proved over the last decade while explaining the anticancer effects of riproximin against target cancer cells. These anticancer effects include induction of apoptosis, mitochondrial stress, cell cycle arrest, inhibition of colony formation and anti-migratory effects [9-11]. In present study, we decipher molecular changes (expressional modifications in cell cycle relevant genes) lying behind already observed cytostatic effects imposed by riproximin in breast and colorectal cancer cells [10, 11].

At first step, previously known cytotoxic effects of riproximin against breast and colorectal cancer cells were re-confirmed by MTT dye reduction. Riproximin induced significant anti-proliferative effects (IC_{50}<1.2ng/ml, 24 hours) in selected breast and colorectal cancer cells (Table 2). The toxic effects were reduced overtime as shown by higher IC_{50} values for 48- and 72-hour time intervals. This reduction in riproximin effects overtime could be due to instability of test compound at standard incubation conditions during longer time intervals and/or development of resistance mechanisms by target cells. Nevertheless, primary breast cancer cells (MCF-7) were almost ten times more sensitive towards riproximin exposure as compared to the metastatic cells (MDA-MB-231). In contrast, both primary and metastatic CRC cells were almost equally responsive towards the test compound (Table 2). Overall, riproximin showed substantial toxic effects and confirmed the previously published data [10, 11].

To understand the mechanistic basis of cytotoxic effects imposed by riproximin in the target cancer cells, three S-phase related genes (CDC6, MCM3 and MCM5) were profiled while exposing the primary and metastatic breast and colorectal cancer cells with three different concentrations of riproximin for 48 hours. The genes selected in this study are linked with the initiation of DNA replication and maintaining the replication forks during the cell cycle [14, 15]. The experiments were performed to investigate concentration dependent effects of the test compound. Riproximin induced concentration dependent effects on the selected three genes in different cancer cell lines (except SW480 cells) as shown in Figure 3 and 4. This in turn indicates that riproximin affects the target cells (at gene levels) regardless of their different molecular subtypes or origin. Based on these findings, it can be suggested that riproximin is an exploitable anticancer compound against primary as well as metastatic phases of breast cancer and colorectal cancer. Riproximin is a significant cytostatic agent and alters the expressional profile of multiple cell cycle related genes in target cells. Further detailed studies (*in vitro* and *in vivo*) are recommended to understand antineoplastic effects of riproximin to support its clinical utilization in future.

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Author Contributions: SSA performed the experiments, MSA drafted the manuscript.

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.