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Research Article

Riproximin mediated cytotoxicity and expression modifications in lung cancer cells

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Abstract

Background: Worldwide, lung cancer is the leading malignancy and responsible for ~20% of cancer related deaths. Evidence from clinical data shows an objective response from currently available therapeutic approaches, especially against advance stages of lung cancer, which in turn, highlight the need to identify novel treatment modalities with better efficacy and lesser off target effects. A ~60kDa active protein component was purified from of the plant *Ximenia american*. The purified protein (riproximin) has shown cytotoxic effects over the years against cancer cells and in the animal models.

Methods: Effects of riproximin on cell viability were investigated by MTT dye reduction methodology. Briefly, the lung cancer cell line (H1299) was cultured in 96-well plate and treated with different concentrations of riproximin (0.39-25ng/ml) for three different time points (24, 48 and 72 hours). After each time interval, MTT solution was added, and concentration of viable cells were determined by ELISA plate reader. Later, the impact of riproximin on selective genes were analyzed by real time PCR strategy. The data was compared with the expression profile of untreated control cells, and fold changes were calculated via Livak method.

Results: The cytotoxic effects of riproximin were time and concentration dependentas more prominent toxicity was observed with increasing concentrations especially for late time intervals (48 and 72 hours). At highest concentration (25ng/ml), the maximum growth inhibition was observed (85%) after 72 hours. Substantial riproximin mediated expressional modulations in related genes were shown by real time PCR. Specifically, CCND1 and CASP3 genes were inhibited, while SERTAD1 and GADD45A were induced in response to riproximin exposure.

Conclusion: Riproximin is a substantial cytotoxic compound and induces prominent anti-proliferative effects in lung cancer cells. Furthermore, the compound has potential to alter the expressional profile of genes. The current study provides vital data about antineoplastic effects of riproximin against lung cancer cells and will provide a platform for further studies and clinical utilization of this protein to treat lung cancer overtime.

Key Words: Riproximin, Ribosome inactivating protein, Lung cancer, Cell line, Cytotoxic, Expression

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INTRODUCTION

It is expected that by 2030, new cancers cases will be ~21.7 million and 13 million affective personal will die due to progression of the disease [1]. Lung cancer is a major cancer and is responsible for ~20% of all cancer related deaths [2]. Regardless of significant progress in oncology, the outcomes of lung cancer treatment are still unsatisfactory, primarily due to late diagnosis, patient age, complexity of the disease which ultimately led restricted therapeutic options [3]. Evidence from clinical trials has shown that cytotoxic therapy can generate limited responses [4]. The existing challenges to treat lung cancer demands for more effective modalities with minimal side-effects and plants can be a rich source of such entities in this context.

Riproximin is a type II ribosome inactivating protein (RIP), a potent cytostatic protein with remarkable anti-proliferative effects on cancer cells. Almost twenty years ago, this protein was extracted from a plant *Ximenia americana* [5]. The cytotoxicity of riproximin was tested against different cell lines and showed notable antineoplastic activity. Riproximin also showed anticancer effects in colorectal and pancreatic cancer liver metastasis rat models. At molecular levels, riproximin regulates several signaling cascades to induce apoptotic and cytostatic activity in human breast and colorectal cancer cell lines. Intensive colony formation and migration inhibition was determined in cell lines because of riproximin treatment. Molecular studies by qRT-PCR methodology revealed up-regulation of ER-stress-

related GADD genes and anticancer cytokine IL24/MDA-7, which play their role in apoptosis [6, 7]. In the current study, cytotoxic effects of riproximin were assessed by MTT dye reduction assay against lung cancer cells (H1299). Furthermore, the impact of this protein on multiple related genes was investigated via real-time PCRs. Overall, the data showed that the riproximin has substantial anti-proliferative and gene modulation potential. The findings will draw intentions of the scientific community to validate riproximin against lung cancer for clinical utilization.

METHODS

Cell Culture

Human lungs cancer cell line (H1299), obtained from ATCC, was cultured in RPMI-1640 medium at standard incubation conditions. In every 48 hours, substantial growth of cells (>80% confluence) was observed. Afterwards, the cells in culture flask were washed with 1X PBS, detached from plate by treating with 0.05% trypsin- EDTA solution (Gibco, Cat#2187277). Obtained cell fractions were transferred to falcon tubes and were centrifuged at 1500rpm for 5 min to collect cell pellets. Supernatant was discarded followed by re-suspension of the cells in new media and transfer to a new culture flask ($25cm^2$) for the next 48 hours. All the following experiments were performed at least in triplicate.

Cytotoxic Assay

MTT dye reduction assay was performed to observe the toxic effects of riproximin on the lung cancer cells (H1299). For this purpose, H1299 cells were cultured in RPMI-1640 medium at standard cell culture incubation conditions. For MTT assay, 4000 cells/well were seeded in 96-well plates as per pre-optimized cell densities determined by growth curve. The cells were treated with different concentrations of riproximin (0.39-25 ng/ml) for 24, 48 and 72 hours. Following the treatment, MTT solution (10mg/ml in PBS) was added (10µl/well) after each time point i.e., 24, 48 and 72 hours. Cells were incubated further at cell culture standard incubation conditions for three hours. Formazan crystals, produced by viable cells, were liquefied by adding 50µl/well of DMSO and optical densities were determined by ELISA. IC values of riproximin were identified by the help of software called GraphPad Prism 6. Untreated cells, grown in parallel, were used as controls in all of these and subsequent experiments of this study.

RNA Extraction and Quantification

The cells were exposed to identified IC concentrations (IC25 and IC50) of riproximin, and RNA was extracted from the treated cells by using a commercial extraction kit (ThermoFisher, Cat#K0731) and quantified by using Nanodrop technology (ND 2000). Nanodrop measurements identified the quality and quantity of the extracted RNA.

cDNA Synthesis and Verification

RNA (1500ng/µl/sample) was used to synthesize cDNA by using ThermoFisher kit (Cat#K1622). Synthesized cDNA was verified by PCR based amplification of reference gene (HPRT1) for the three samples (control, IC25, IC50) in triplicate. Amplified products were visualized on 2.5% agarose gel by electrophoresis methodology. Four genes were selected, and primers were designed by using Primer3Plus software (Table 1)

Real Time PCR

Four selected genes (CASP3, CCND1, GADD45A, SERTAD1) were amplified by Real-Time PCR while using the three cDNA samples (untreated control, IC25 and IC50 treated). Amplification of a reference gene (HPRT1) was used to normalize the data.

Data Analysis

Categorical data generated from cytotoxicity assays was presented as frequency percentages. In case of real-time PCR analysis, fold changes were calculated by Livak $2^{-\Delta\Delta CT}$ method by comparing Cq values of experimental (riproximin treated) and untreated control samples.

Table 1: Primer sequences for selected genes

GeneName	Primers		Product Size (bp)
CASP3	F	GAGGCCGACTTCTTGTATGC	195
	R	AGCGTCAAAGGAAAAGGACT	
CCND1	F	GGGGGCGTAGCATCATAGTA	114
	R	GTGGTGGCACGTAAGACACA	
GADD45A	F	AACGGTGATGGCATCTGAAT	127
	R	CCCTTGGCATCAGTTTCTGT	
SERTAD1	F	TCGACTCCTGGTGGCTAGAT	263
	R	GTCCGAGCTTGCCAGTAAGT	
HPRT1	F	GACCAGTCAACAGGGGACAT	164
	R	CTTGCGACCTTGACCATCTT	

RESULTS

Cytotoxic Effects of Riproximin

Treatment with riproximin inhibited the proliferation of lung cancer cells. Formazan crystals produced different shades of purple after adding DMSO, lighter shade of purple, shows less percentage of survival. The effects were calculated numerically as percentages of untreated controls via GraphPad Prism 6 software and are shown in Figure 1. The decline in bar graphs reflects decreased optical density by ELISA reader, which in turn reflects less percentage of survival. Reduction in proliferation was gradual in the cells especially for later time intervals (48 and 72 hours) when exposed to riproximin. Overall, the effects of riproximin were time and concentration dependent, which means the inhibitory effects were observed to be more prominent with increasing concentrations of riproximin especially for late time intervals (48 and 72 hours). At highest riproximin concentration (25ng/ml), the minimum inhibition of proliferation (46%) was observed after 24 hours exposure time, and the maximum inhibition (85%) was observed after 72 hours exposure time as shown in Figure 1.

RNA Extraction and cDNA Verification

Lung cancer cells (H1299) were cultured in 6-well plates (200,000cells/well/2ml) and were treated with two inhibitory concentrations of riproximin (IC25, IC50) for 48 hours. After 48 hours, cells were collected, and total RNA was extracted from untreated and treated H1299 cells. RNA was quantified by Nanodrop ND 2000. The extracted RNA was of good quantity (79-449ng/µl/sample) and quality as reflected by 260/280 ratios (2.03-2.05) in Table 2. cDNA was synthesized from extracted RNA and agarose gel electrophoresis methodology was used to visualize the amplified product of samples as shown in Figure 2.

Optimization of Primers

The designed primers for selected genes (CCND1, GADD45A, CASP3 and SERTAD1) were optimized by gradient PCR methodology using three different annealing temperatures (58, 60 and 62°C). The amplified products showed specific amplification of the selected genes at all checked temperatures as shown in Figure 3.

Expression Modification in Genes

Using the two concentrations of riproximin (IC25 and IC50) led us to observe the concentration dependent effects of this protein on lung cancer cells (H1299). For this purpose, cDNA samples (control, IC25 and IC50) were used for real-time PCR based experiment while using SybrGreen master mix along with gene specific primers. In response to treatment with two different concentrations of riproximin (IC25: 1.3ng/ml, IC50: 7.6ng/ml), both CASP3 and CCND1 genes showed similar patterns where maximum inhibition was observed with lower concentrations of the protein. In contrast to this, SERTAD1 and GADD45A genes showed more up-regulation at higher concentrations of riproximin. To summarize, CASP3 and CCND1 did not follow the concentration dependent inhibition in expression, while SERTAD1 and GADD45A responded more effectively to increasing amount of riproximin exposure (Figure 4).

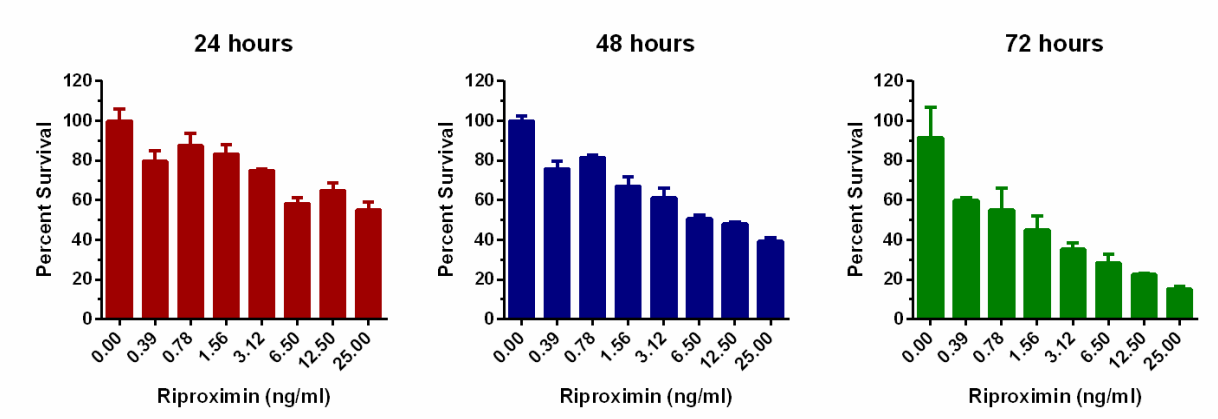

Figure 1: Inhibition of proliferation in H1299 cells. The cells were exposed to riproximin and resulting toxicity was monitored by using MTT dye reduction assay.

Table 2: RNA Quantification and Quality

Cells	Treatment	RNA (ng/µl)	260/280
H1299	Control	448.7	2.05
	1.3ng/ml (IC25)	171.3	2.05
	7.6ng/ml (IC50)	79.8	2.03

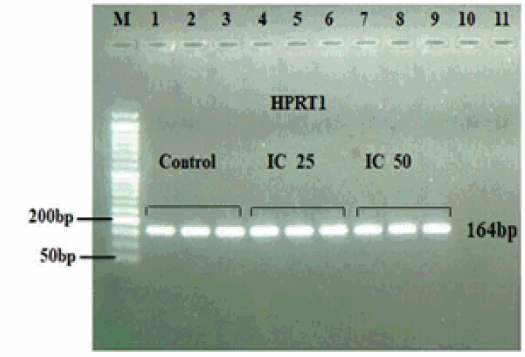

Figure 2: cDNA verification was achieved by applying conventional PCR method and amplification of a reference gene (HPRT1) followed by agarose gel electrophoresis.

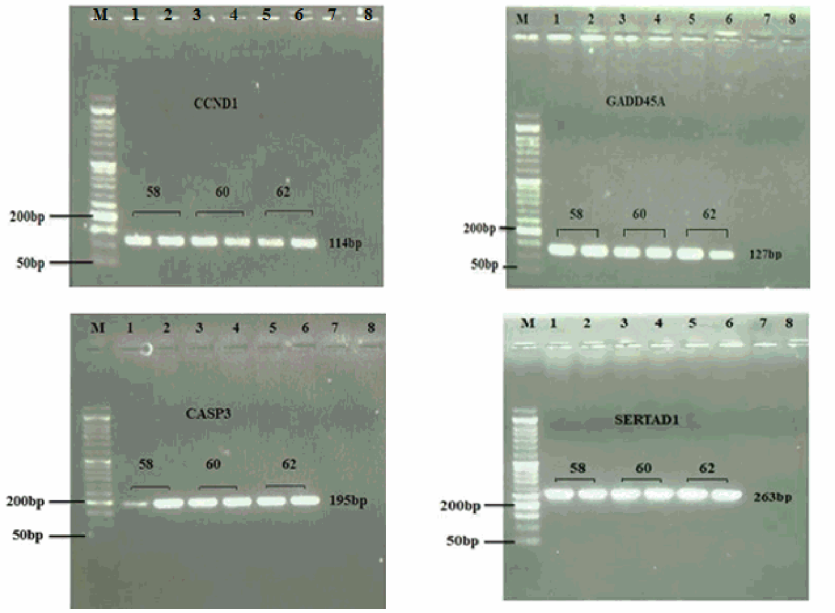

Figure 3: Primer optimization was done by gradient PCR strategy by using a verified cDNA from untreated control sample and agarose gel electrophoresis method.

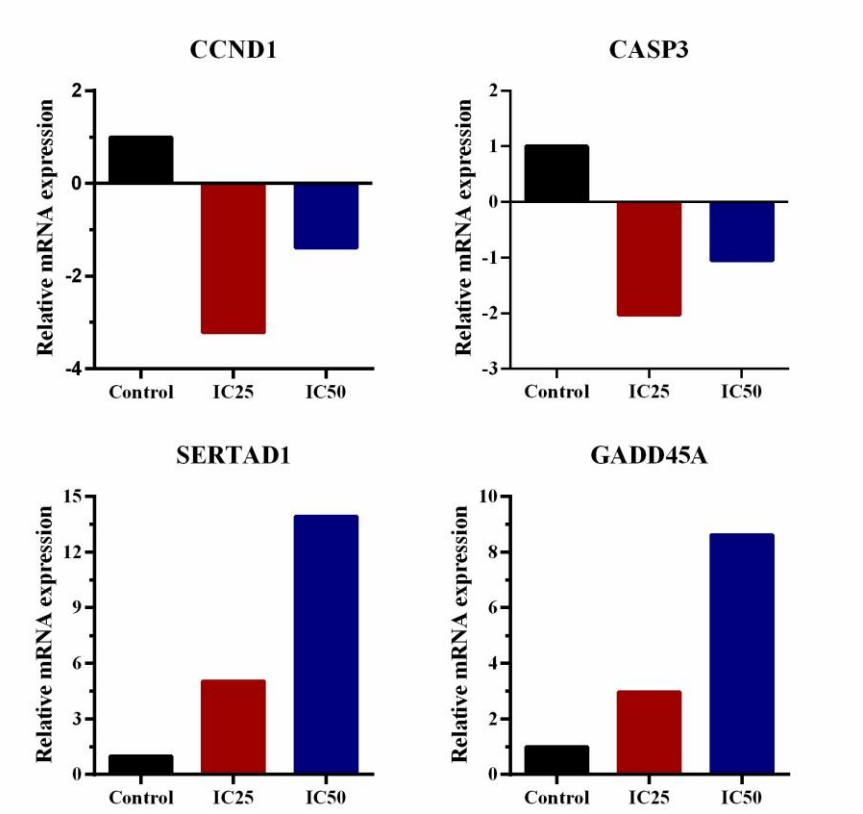

Figure 4: Real Time PCR analysis was done by using the treated and untreated samples and expression profiling by using gene specific primers. The Livak method was used to find the fold changes in the groups.

DISCUSSION

X. americana is a tropical plant and has been used by the local healers of African countries to treat different diseases including cancers. Back in 2006, an active fraction of this plant was extracted from the seeds of this plant. The purified active fraction was named as riproximin. Since its discovery, this protein has been tested against several cancer cell lines for its antineoplastic potential [8]. Over time, it has been shown that riproximin has substantial anticancer potential and can inhibit the proliferation, migration, colony formation of cancer cells [6, 7]. Riproximin is a very important anticancer naturally occurring protein and needs attention to develop strategies for its clinical utilization. For instance, it is also to be remembered that the closest relative of riproximin is ricin, which is being used clinically to treat cancers. However, to increase the possibility of riproximin clinical utilization, scientists must devote more efforts to understand the biological and molecular effects of this protein on cancer cells, as these data will be required before entering clinical trials. Continuous cell cycle is an important feature of cancer cells, and several cytotoxic/cytostatic agents are being produced continuously as therapeutic entities against cancers. Considering this, present study was aimed to understand the impact of riproximin on cell cycle of lung cancer cells.

The anticancer role of riproximin believed to be partially due to cell proliferation inhibition. Many *in vitro* studies showed the anti-proliferative effects of riproximin on different cancer cell lines including breast cancer, colorectal cancer, pancreatic cancer and cervical cancer [6-8]. For this reason, the anti-proliferative effects of riproximin were studied as first step. For this purpose, MTT assay was performed where 4000 cells were seeded in each well of 96-well plates followed by treatment with different concentrations of riproximin for different time period (24, 48 and 72 hours). The effects were calculated numerically as percentages of untreated controls via GraphPad Prism 6 software. We observed that the anti-proliferative effects of riproximin were time and concentration dependent against the lung cancer cells. Concentration dependent effects mean, the inhibitory effects were observed more toxic with increasing concentrations of riproximin especially for late time intervals (48 and 72 hours). At highest riproximin concentration that was 25ng/ml, a maximum inhibition of 85% was observed after 72 hours. This in turn shows that cells become more responsive towards riproximin with the passage of time while showing no resistance overtime. This is an important piece of information, and if authenticated in

clinical scenarios in similar patter, lung cancer patients would be treated with higher dose of riproximin without development of resistance for longer time periods. In a previously published *in vitro* study, similar effects of riproximin were observed on breast and colorectal cancer cell lines where riproximin showed remarkable concentration and time dependent anti-neoplastic results in breast cancer cell lines [6-8]. Overall, toxicity assay reflected that riproximin can inhibit the proliferation of lung cancer cells effectively and it was comparable with other cancer cell lines as per published data. After observing cytotoxic and anti-proliferative results of riproximin on lung cancer, the next step was to evaluate its impact on expressional modulation via Real-time PCRs. For this purpose, lung cancer cells (H1299) were cultured (200,000cells/well/2ml media) and treated with two inhibitory concentrations of riproximin (IC25, IC50) for 48 hours and untreated cells were used as control. Total RNA was extracted, and cDNA was synthesized and confirmed by PCR based amplification of a reference gene (HPRT1), Primers against four genes (CCND1, CASP3, SERTAD1, GADD45A) were designed and real-time PCRs were performed to evaluate the expressional alterations. CASP3 and CCND1 did not follow the concentration dependent inhibition in expression, while SERTAD1 and GADD45A responded more effectively to increasing amount of riproximin exposure. However, detailed investigations are needed to understand the precise nature of effects being induced on cell cycle genes via riproximin. Nevertheless, riproximin was able to alter the expression of cell cycle (CCND1, SERTAD1) and apoptosis (CASP3, GADD45A) related genes in lung cancer cells. In conclusion, the compound showed anti-proliferative effects in lung cancer cells. At same time, it showed potential to change expression of important genes. Further investigations about understanding the molecular mechanisms associated with riproximin and potential application methods should be explored.

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