Cancer Research and Medicine
DOI: https://doi.org/10.63626/0m87x035
ISSN Online: 3104-4131
ISSN Print: XXXX-XXXX

Research Article

Ribosome inactivating plant protein mediated regulation of transcriptomic profile in liver cancer cells

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Abstract
Background: Hepatocellular carcinoma (HCC) is the primary malignancy of the liver that accounts for about 90% of liver cancers. Utilization of natural compounds could be instrumental for HCC patients with better therapeutic efficacy and lesser side effects. A plant based ribosomal inactivating protein (riproximin) has shown substantial antineoplastic properties against the variety of cancer cell lines. The purpose of this study was to investigate the cytotoxic effects of riproximin on cell proliferation of liver cancer cells and to determine the expressional changes induced in cell cycle and cell stress relevant genes.
Methods: The HepG2 cell line (liver cancer cells) was cultured in under suitable conditions and exposed to different concentrations of riproximin (0.38–50ng/ml) for 24-72 hours followed by cytotoxicity analysis. Afterwards, the cells were exposed to riproximin (1-20ng/ml) for 48 hours and RNA was extracted using a commercial extraction kit. RT PCRs were carried for analysis of cell cycle (CCND1, CDKN1A) and cell stress (GADD45A) relevant genes. Fold changes were calculated by Livak $2^{-\Delta\Delta CT}$ method by comparing Cq values of experimental (riproximin treated) and untreated control samples.
Results: Findings revealed concentration and time dependent inhibitory effects of riproximin on liver cancer cells. The growth inhibition was maximum at 72 hours with 50ng/ml concentration. RT PCRs results showed that riproximin inhibited the expression of cell cycle promoter gene (CCND1), while cell cycle inhibitor (CDKN1A) and cell cycle inducers (GADD45A) were up regulated.
Conclusion: Riproximin harbors significant cytotoxic effects against liver cancer cells. Expressional variations in multiple related genes are imposed by riproximin in the liver cancer cells.
Key Words: Ribosome inactivating protein, Plant, Cancer cells, Cell cycle, Cell stress

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INTRODUCTION

Cancer is a gathering of unusual cellular development with potential to spread to different parts of the body. Hepatocellular carcinoma (HCC) is 5th most successive neoplasm worldwide yet, attributable to the absence of viable therapy choices, addresses the third driving reason of cancer death. Malignant growth starts in the liver and is a forceful cancer that oftentimes happens in the setting of on-going liver illness and cirrhosis [1]. Current treatment of liver cancer includes four most used therapeutic approaches such as surgery, chemotherapy, targeted therapy and immunotherapy [2]. The principal objective of these therapies is to eliminate liver tumor and to reduce the proliferation of cancer cells. For liver disease patients, new treatment alternatives are important. Utilization of natural compounds as well as nanotechnology might give patients better results with lower toxicity and less side effects [3]. The main therapeutic approaches for HCC are careful surgical resection or liver transplantation, but surgical procedure is not an option for most patients. Chemotherapeutic treatment is inadequate against HCC, and no single medication drags out survival successfully. The circumstance requests to assess novel mixtures for compelling therapy of liver cancer [4, 5].

Cell division is a firmly managed process of events including various check points that evaluate extracellular development signals, cell size, and DNA integrity. The mammalian cell cycle involved five consecutive stages: G0, G1, S, G2 and M. Movement all through the cell cycle depends on the various cyclin binding complexes that keep cells from going into another stage until they have effectively completed the previous stage. The vital participants in regulation of cell cycle are the cyclin-dependent kinases (CDKs), a class of serine/threonine kinases. During the cancer cell transformation, deregulation of cell cycle is considered as a hallmark. Cancer cells

avoid normal cell cycle routes via alterations in regulatory networks and lead to uncontrolled cellular growth. Thus, it is of paramount importance to investigate such alterations in cell cycle of cancer cells. Furthermore, investigating the natural/synthetic compounds to subvert these changes is a promising domain to identify novel therapeutic options for cancer treatment [6, 7].

Among the naturally occurring therapeutic agents, plants gained special importance for the treatment of diverse types of cancer. In different areas of Africa, kernels of the *Ximenia americana* plant are being used as a traditional medication for different types of cancer [8, 9]. Active fraction from the seeds of this plant was extracted and named as riproximin. The protein is a profoundly toxic protein with critical antineoplastic potential against many cancer cell lines. Riproximin cytotoxicity was tested in the past against different types of cancers and showed a significant anti-proliferative movement. Further examinations in colorectal and pancreatic disease liver metastasis rat models likewise settled the huge capability of riproximin as an anticancer drug *in vivo* [10-13].

For cancer treatment, the main aim is to suppress the proliferation of cancer cells by inducing arrest in cell cycle. Various cytostatic drugs are being developed for the treatment of liver cancer. These cytostatic agents are particularly important in a way that they halt the cell division of fast proliferating cancer cells dominantly and spare the normal cells, thus induce minimal side effects. At molecular levels, riproximin regulates multiple signaling cascades prompting cytostatic and apoptotic impacts in human malignancy cells. Significant hindrances of migration and colony formation were likewise seen in cancer cell lines considering riproximin exposure. A significant arrest in S phase and nuclear fragmentation were observed in a previous study [13, 14].

In proposed study, intentions were to investigate riproximin mediated cytotoxic effects in liver cancer cells. In addition, effects of the selected plant protein were studied on cell cycle and stress related genes in liver cancer cells.

METHODS

Cell Culture

Human liver cancer cell line (HepG2) was cultured in RPMI-1640 medium supplemented with L-glutamine (2mM), 10% fetal bovine serum (FBS), streptomycin (100µg/ml) and penicillin (100IU/ml). Standard incubation conditions (37°C in humidified air with 5% CO_2) were maintained for growth of the cells.

Growth Curve Analysis

To optimize cell densities for the experiments, cells were cultured in 96-well culture plates (1000-6000 cells/well/100µl medium) for 24-72 hours. MTT solution (10mg/ml) was added (10µl/well) after each point and formed crystals of formazan by the viable cells were dissolved by adding 50µl/well of DMSO. Optical densities were determined by an ELISA plate reader (540/690 nm) and growth curves were generated with time intervals on X-axis and absorbance values on Y-axis.

Toxicity of Riproximin

MTT assay was performed to observe the toxic effects of riproximin on liver cancer cells. For this purpose, the cells (4000 cells/well) were seeded in 96-well plates and were treated with riproximin (0.38-50ng/ml) for 24-72 hours (Table 1). Following the treatment, MTT solution (10mg/ml in PBS) was added (10µl/well) and the same procedure was repeated as described in growth curve analysis for measurement of viable cell population. Untreated cells, grown in parallel, were used as controls in all the experiments of this study.

Expressional Profiling Steps

Liver cancer cells were cultured in 6-well plates (150,000 cells/well) and the next day were exposed to riproximin (1-20ng/ml) for 48 hours. Afterwards, total RNA was extracted from treated and untreated cells (control) by using commercial kit (Thermo Fisher Scientific, Cat#K0731). Quantity and quantity of the RNA was determined by Nanodrop technology followed by cDNA synthesis step, while using a commercial kit (Thermo Fisher Scientific, Cat#K1622) along with the extracted RNA (500ng/sample). Amplification of a reference gene (HPRT1) was carried out to verify the synthesis step. A total of 10µl/sample of the amplified products were loaded on 2.0% agarose gel and visualized by ethidium bromide staining. Primers for three selected genes (CCND1, CDKN1A and GADD45A) were designed by using Primer3Plus software (Table 2) and were optimized by gradient PCR methodology. Again, amplified products from the primers were visualized on 2.0% agarose gel electrophoresis.

Real Time PCR

Confirmed cDNA samples from above step were used for expression profiling of the three genes (CCND1, CDKN1A, GADD45A). By using SybrGreen master mix along with cDNA samples, amplification procedures were carried out for the selected genes. Amplification of a reference gene (HPRT1) was used to normalize data. Expression of untreated cells was used as control and experiments were executed in triplicate.

Statistical 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. For this purpose, obtained Ct values of control and treated samples were normalized with Ct values of respective reference gene values. Afterwards, the difference between normalized Ct of treated and control samples were converted into fold change differences.

Table 1: Treatment of liver cancer cells with riproximin for MTT assay

Cell Line	Riproximin Concentration (ng/ml)
HepG2	0.38, 0.76, 1.56, 3.12, 6.25, 12.5, 25, 50

Table 2: Primers designed for expression profiling

Gene	Primer Sequence (F.)	Primer Sequence (R.)	Product Size
CCND1	GGGGGCGTAGCATCATAGTA	GTGGTGGCACGTAAGACACA	114
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA	260
GADD45A	AACGGTGATGGCATCTGAAT	CCCTTGGCATCAGTTTCTGT	127
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT	164

RESULTS

Growth of Liver Cancer Cells

To optimize cell densities for subsequent experiments, the cells were cultured in the 96-well culture plates (1000-6000 cells/well/100µl medium) for 24-72 hours. MTT solution was added and crystals of formazan by the viable cells were dissolved by adding 50µl/well of DMSO. Optical densities were determined by an ELISA plate reader (540/690 nm) and growth curves were generated with time intervals on X-axis and absorbance values on Y-axis. This data led to the identification of optimal cell numbers to be used in the experiments. The selected optimal number of cells to be used (for cytotoxic analysis) was 4000 cells/well for 48 hours, as this number shown the continuous and highest linear growth throughout the three-time intervals (Figure 1). Further increase in number (5000-6000/well) led to decline of growth curve overtime (72 hours) probably due to space constraints, accumulation of wastes and scarcity of available nutrients.

Riproximin and Cytotoxic Effects

Human liver cancer cell line (HepG2) was allowed to grow in RPMI-1640 cell culture medium overnight and treated with increasing concentrations of riproximin (Table 1). The inhibitory effects were assessed by MTT dye reduction assay. For this purpose, the cells were seeded in 96-well plates at pre-optimized cell densities and were treated with riproximin (0.38-50 ng/ml) for 24-72 hours. After the treatment intervals, MTT assay was performed as described in the method section. The results showed that in response to riproximin exposure, there was a clear decline in cell growth. Development of purple color was directly in proportion to the number of viable cells present in a well at given time (results not shown here). The intensity of color was getting lighter with increasing concentration of riproximin, which highlights the growth inhibitory effects of the tested plant protein. The cytotoxic effects were almost concentration dependent as with increasing concentration, there was more prominent inhibition of cellular growth. Furthermore, the effects were time dependent as higher intensity of cell growth inhibition was observed after 72 hours exposure time. All in all, riproximin showed substantial cytotoxic effects against the liver cancer cells and inhibited cell proliferation (Table 3).

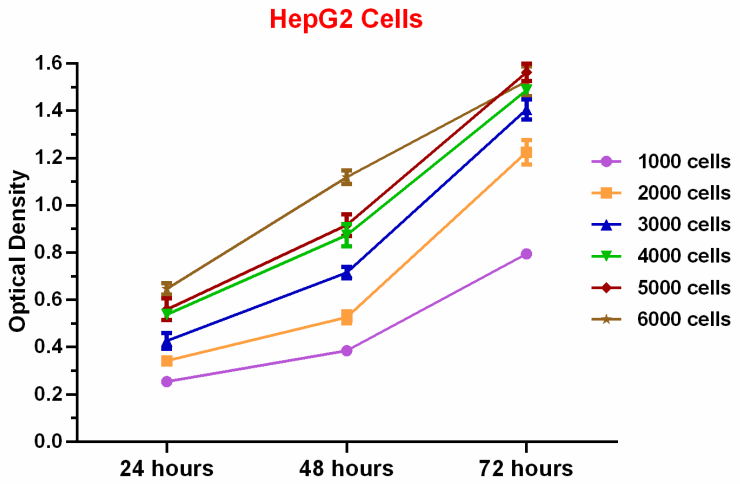

Figure 1: Growth curve with time intervals on X-axis and absorbance values on Y-axis.

Table 3: HepG2 cells survival after riproximin treatment

	Riproximin concentration (ng/ml)								
	0.0	**0.38**	**0.76**	**1.56**	**3.12**	**6.25**	**12.5**	**25**	**50**
24 hours	100	102.7	86.7	85.5	76.4	61.1	52.0	40.2	34.7
48 hours	100	89.7	76.6	60.5	47.6	38.3	36.5	32.4	32.7
72 hours	100	89.5	63.1	39.3	17.1	14.7	26.9	7.3	4.8
	Cell survival (% of untreated controls)								

RNA Extraction, cDNA Verification and Primer Optimization

Total RNA was extracted from the collected cell pallets of cultured cells of HepG2 cell line by using commercially available RNA extraction kit and it was quantified by using nanodrop technology. Considering an initial culturing of 150,000 cells/well/2ml media in a 6-well culture plate, a substantial amount of RNA was extracted as shown by 82ng/µl in eluted volume. However, from the samples treated especially for 20 ng/ml group, the RNA extracted was low in quantity (34ng/µl) because of growth inhibition and cytotoxic effects imposed by riproximin and availability of lesser cell number at the end. A ratio of 260/280 obtained by NanoDrop is a benchmark to monitor the quality of extracted RNA and a value 1.9-2.1 shows the good quality of the RNA. In these experiments, 260/280 ratio was between 2.0-2.06 which reflects a good quality of extracted RNA as shown in Table 4. It was a necessary step to check the integrity of extracted RNA before starting the real-time PCR based expression profiling of cell cycle related genes. For this purpose, RNA extracted from controls and treated samples were used to synthesize the cDNA and it was verified by PCR based amplification of a reference gene (HPRT1). To visualize the amplified product of samples, agarose gel electrophoresis was performed. Amplified samples loaded on agarose gel showed the good quality of cDNA as single specific bands of the reference gene (HPRT1) was observed (Figure 2). Primers were designed for selected genes (CCND1, GADD45A, CDKN1A). Gradient PCR methodology was used to optimize the primers by using three different annealing temperatures (56, 59 and 62°C). The amplified products showed specific amplification of the selected genes at all temperatures as shown in Figure 3.

Table 4: RNA concentrations obtained by nanodrop technology.

Sr. No	Treatment	RNA Conc. (ng/µl)	260/280
1	Control	82.8	2.05
2	1 ng/ml	83.4	2.06
3	5 ng/ml	93.0	2.05
4	20 ng/ml	34.3	2.00

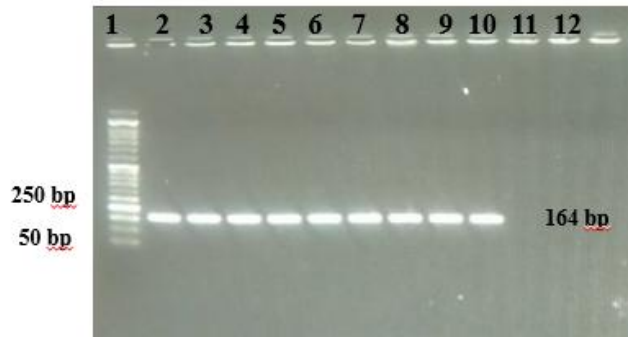

Figure 2: Gel electrophoresis of amplified cDNA of HPRT1 gene from HepG2 cells for cDNA verification. Sample distribution (left to right): Well 1: DNA marker (50bp) Well 2-4: HepG2 untreated control, Well 5-6: HepG2 treated with riproximin 1 ng/ml, Well 7-8: HepG2 treated with riproximin 5 ng/ml, Well 9-10: HepG2 treated with riproximin 20 ng/ml,Well 11-12: Negative control.

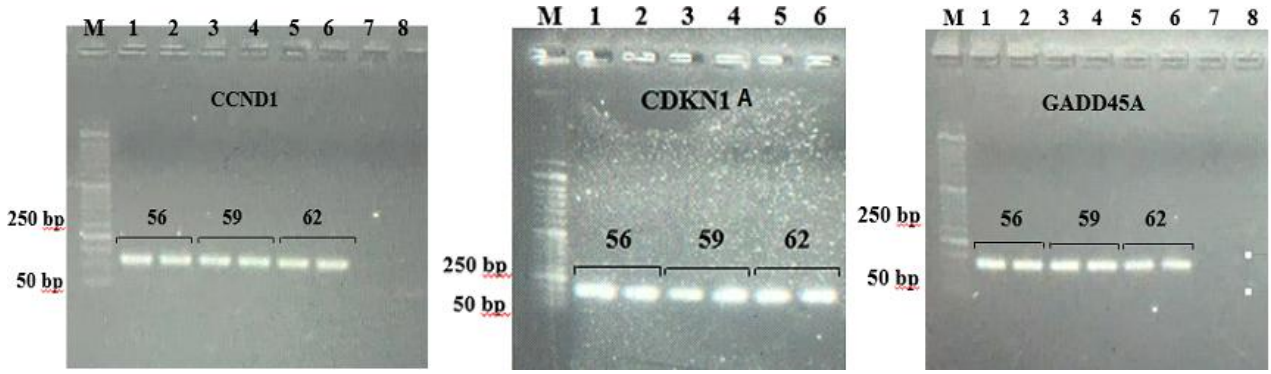

Figure 3: Gradient PCR, sample distribution (left to right) for amplification of CCND1, CDKN1A and GADD45A genes. Well M: DNA marker (50bp), Well 1-6: amplification at three different temperatures, Well 7-8: Negative control.

Expression Modulations by Riproximin

Real-Time PCR was performed by using SybrGreen master mix for selected 3 genes by using the cDNA samples (control, 1, 5 and 20 ng/ml treated) in triplicate. The results showed that the two of these genes (GADD45A, CDKN1A) were up-regulated on all checked concentrations of riproximin (1, 5, 20 ng/ml), while down regulation was seen in CCND1 expression only with the highest concentration (20ng/ml) of riproximin.

In selected liver cancer cell line, riproximin induced moderate expression of CCND1 at lower concentrations of riproximin (1 and 5ng/ml), while at higher concentration of (20ng/ml), inhibition (-3.63-fold) of CCND1 gene was observed as seen in Figure 4. In selected liver cancer cell line, riproximin induced moderate expression of CDKN1 gene at lower concentrations (1 and 5ng/ml), while a noticeable induction (7.27-fold) at higher concentration (20ng/ml) was observed. Riproxamin induced moderate expression of GADD45A gene at lower concentrations (1 and 5ng/ml) in the selected cell line (HepG2) was witnessed, while an abrupt induction (29.9-fold) was observed at higher concentration (20ng/ml). Overall, riproximin induced the cell cycle inhibitor (CDKN1A) and stress inducer (GADD45A).

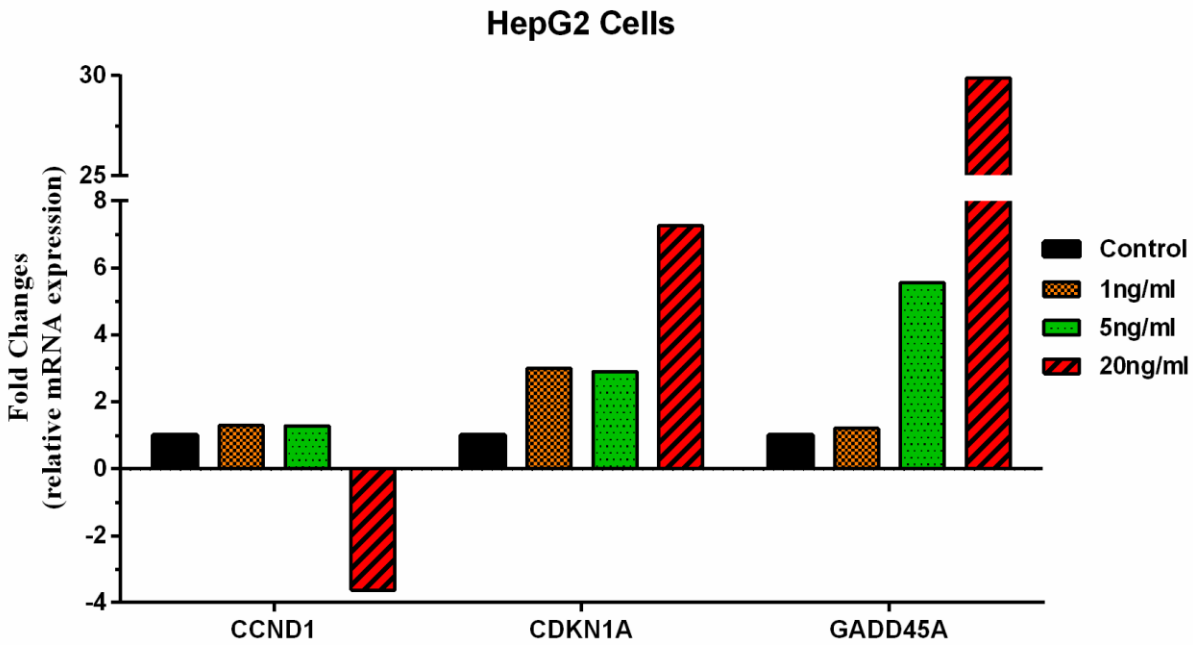

Figure 4: Real time PCR of selected genes. The cells treated with riproximin, and expression was monitored by real-time PCR technique. Fold changes reflected marked changes in the genes after exposure to the protein.

DISCUSSION

HCC is a lethal cancer with life expectancy less than 3 years in developed and even less in developing countries [15]. The most commonly found risk factors are: chronic hepatitis B and C, obesity, diabetes (type 2), cirrhosis (excessive alcohol use), NAFLD (non-alcoholic fatty liver disease), aflatoxins and smoking [16]. There are treatment options for HCC like partial liver resection, liver transplantation and chemotherapy but numerous trials are progressing to asses new drugs with lesser side effects and more effectiveness at advance stage of the disease [17].

Ribosome inactivating proteins (RIPs) gave off an impression of being an extraordinary exploration interest because of their expected use in cancer treatment. A few RIPs show vigorous toxicity towards cancer cells and low toxicity towards normal cells, they block or repress cancer development generally through apoptosis [18]. RIPs are distinguishing catalytic proteins, which repress protein translation irreversibly in their objective eukaryotic cells by changing the 28S rRNA subunit. There are different classes of RIPs and riproximin, the protein of interest at this stage, belongs to type II RIP class. The advancement in new anticancer medications is a remarkable issue and utilization of plants is possibly a great source of data for distinguishing the latest particles with antineoplastic action. For certain cancerous cell lines, riproximin is considered as a newly distinguished cytotoxic RIP with high specificity. Its action was perceived as the principal part in powder of the plant utilized by Africans for treatment of the disease. The powder was obtained from plant *X. americana* and it was investigated by the gene sequencing of ribulose bisphosphate carboxylase. The cDNA arrangement of riproximin protein was demonstrated to contain a single A and a B-chain individually. The cytotoxic mechanism of action was found to be dependent on cell take-up of riproximin followed by the A-chain induced depurination of the 28S ribosomal RNA and elicitation of uncoiled protein reaction. The distinctiveness of riproximin relied upon the B-chain associated bond to the cell surface glycans, which presence is significant for later incorporation into cells and cytotoxicity [8].

In this study, effects of riproximin on cell cycle and stress related genes were evaluated. Firstly, MTT dye reduction assay was performed to observe the toxic effects of riproximin on the liver cancer cells. It was observed that growth inhibition was there at different time intervals and concentrations applied. At 72 hours with 50ng/ml concentration (highest dose), maximum anti-proliferative effects were observed. Thus, riproximin showed concentration and time dependent effects on liver cancer cells, which means with increase in time and concentration, more effective growth inhibition was observed. In a previously published *in vitro* study, similar effects of riproximin were observed on breast cancer cell lines where riproximin showed remarkable concentration and time dependent anti-neoplastic results in breast and colorectal cancer cell lines [13, 14].

Following the exposure period (48 hours) in a sperate experiment, cell pellets were collected from the culture plates and good quality of extracted RNA were used for investigating the expression changes in genes (GADD45A, CCND1, CDKN1). The profiling of these genes was performed by using the cDNA samples treated with riproximin (1, 5 and 20ng/ml) along with SybrGreen master mix and real-time PCR. The results showed that stress inducer gene (GADD45A) and cell cycle inhibitor (CDKN1) were up regulated on all tested concentrations. While down regulation was seen with 20ng/ml concentrations in cell cycle promoter gene (CCND1) as compared to lower concentrations (1, 5ng/ml), which showed up regulation of this gene. This data shows that riproximin can induce cell cycle inhibitors and stress inducers (GADD45A, CDKN1) effectively, while it can inhibit cell cycle promoters (CCND1) at higher concentrations (20ng/ml) in target liver cancer cells. To extrapolate, it can be claimed that riproximin inhibits the cell cycle progression and induces the toxic effects via promoting cellular stress. Overall, it has been confirmed from the data that riproximin has shown cytotoxic potential and is a good cytostatic agent and needs due consideration for future research.

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Author Contributions: UUI and NS performed experimental work, SS prepared figures, MAS wrote the manuscript.
Competing Interests: Authors declare no competing interests.
Ethical Statement: Not Applicable
Financial Support: No specific grant was availed for this research.
Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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