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Research Article

Riproximin mediated effects on transcriptomic profile of PI3K-AKT-mTOR pathway genes in breast cancer cell lines

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Abstract

Background: Riproximin, a type II ribosome inactivating protein, is a vital antineoplastic agent and can be used as alternative to chemotherapy specifically to kill tumour cells. Since discovery, this protein has been tested against various cancer cell lines and animal models to explore its anti-neoplastic activities. In this study, riproximin has been tested to unfold its impact on multiple genes related to PI3K-AKT-mTOR signaling pathway. This pathway has its significant importance in regulation of cellular functions which include metabolism, proliferation, survival and growth of cancerous cells. Due to the valuable importance in carcinogenesis, this pathway is also being targeted for therapeutic purposes.

Methods: Breast cancer cell lines were exposed to different concentrations of riproximin (MDA-MB-231: 1-50ng/ml, MCF-7: 1-20ng/ml) followed by total RNA extraction, cDNA synthesis and expressional profiling of 10 genes related to PI3K-AKT-mTOR signaling by using real-time PCR. Fold changes were identified via Livak method while comparing the data with untreated cells grown in parallel.

Results: Real-time PCR demonstrated a substantial potential of riproximin to alter expression of the genes. Among these targets, the most effective de-regulations in MDA-MB-231 cells were found in FOS (16fold) followed by JUN (6fold) and NFKB1 (4fold). In MCF-7 cells, most substantial modifications were observed in NFKB1 (14fold), CD14 (9fold) and PDK1 (6fold).

Conclusion: Riproximin bears significant cytotoxic potential against primary and metastatic breast cancer cell lines. Substantial expressional modulations in PI3K-AKT-mTOR signaling pathway related genes are imposed by riproximin in the cancer cells. Further detailed *in vitro* and *in vivo* studies are required to understand the precise impact of riproximin exposure on PI3K-AKT-mTOR signaling, which ultimately will pave the way for its clinical utilization.

Key Words: Cancer, Plant Protein, PI3K-AKT-mTOR, Genes, Target

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INTRODUCTION

Breast cancer develops from malignant cells in breast tissue. It is the most prevalent cancer in females and affecting a huge proportion of population. Treatment strategies being utilized to control breast cancer include surgical removal of the tumor mass, chemotherapy, radiations, hormonal therapy and immune modulation [1-4]. Despite the advancements in treatment, options are largely palliative in nature and are only used to mend the worth of life. Challenges in breast tumors treatment are still there especially for advanced stages where 5-years survival rate is not more than 30% [5-8]. A huge burden of side-effects and expensive nature of treatment modalities impose further obstacles to control the disease. Tumor heterogeneity along with complex intracellular pathways in breast cancer cells further adds the speed breakers. Among the many cancerous signaling cascades, PI3K-AKT-mTOR signaling shows a significant role in cellular transformation and proliferation of breast tumors [9]. In cancerous conditions, often this pathway is found to be hyper activated and leads to uncontrolled and continuous proliferation of breast cancer cells. Keeping in view the crucial involvement of PI3K-AKT-mTOR signaling in cancer progression, synthetic inhibitors are being developed against this signaling axis for therapeutic

purposes. At the same time, investigating naturally occurring modulators of PI3K-AKT-mTOR signaling for therapeutic purposes is a promising area of research [10,11].

For many years, plants proved to be a rich source of medicinal compounds and are being explored continuously for their therapeutic efficacy against diseases including cancers [12]. *Ximenia americana* is one of such plant which has been used by the local healers (Hakeem) in African countries to treat cancers. Almost 15 years ago, an active fraction from the crude extracts of this plant were purified and was named as riproximin [13]. This purified protein has been tested against various cancer cell lines for its anticancer effects [14]. Reticence of colony formation, migration, and proliferation of cancerous cells by riproximin is being reported in a previously conducted study [15]. Furthermore, riproximin was shown to induce apoptotic and cytostatic effects in this study. The data compelled us to explore the consequences of this protein on PI3K-AKT-mTOR cascade, the vital signaling pathway for tumor cells.

To validate the sensitivity of breast cancer cells towards riproximin in our experimental settings, MTT assay was performed. Afterwards, riproximin mediated expression modifications in PI3K-AKT-mTOR signaling related genes were identified via real-time PCR methodology.

METHODOLOGY

Cell Culture

Two particular cell lines for breast cancer, MCF-7 and MDA-MB-231, were cultured in Roswell Park Memorial Institute (RPMI)-1640. The media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 IU/ml penicillin. Breast cancer cell lines were incubated and maintained at standard incubation conditions (5% CO2, 37˚C temperature, 100% standard humidified atmosphere). Both cell lines were passaged an average of two to three times per week to maintain the cells' viability and proper development under the specified conditions.

Riproximin Treatment

The cells were seeded in 6-well plates (2000µl media/well) at optimized densities (200000cells/well) and were treated with the protein dissolved in PBS (MDA-MB-231: 1, 10, 50ng/ml, MCF-7: 1, 5, 20ng/ml) for 48 hours. Following treatment intervals, the cell palettes were collected and stored at -80C till further use. Untreated cells grown in parallel were used as controls and experiment was conducted in triplicate.

RNA Extraction and Quantification

Total RNA extraction was performed by using spin column technology and a commercially available RNA extraction kit (Thermo Fisher Scientific, Cat#K0731). A spectrophotometer-based instrument known as the Nanodrop ND2000 was used to quantify the extracted RNA.

cDNA Synthesis and Verification

Following RNA extraction and measurement, a total of 1500ng of extracted RNA/sample was utilized to synthesize 40µl of cDNA using dNTPs, OligodT, and reverse transcriptase (Thermo Fisher Scientific, Cat#K1622). To validate cDNA synthesis procedure, PCR based strategy was used to amplify the reference gene GAPDH. Agarose gel electrophoresis was used to view the amplicon.

Primer Designing and Optimization

Primers of the chosen genes—FOS, PIK3R2, CASP9, JUN, PDK1, CD14, NFKB1, FASLG, CCND1, and CDKN1B—were created by using Primer 3 and selecting gene-specific sequences from the NCBI Gene Bank (Table 1). The gradient PCR technique was used to optimize the chosen primers for the ten genes, testing a range of annealing temperatures, 56–62^{0}C. Utilizing an electrophoresis and 2.5% agarose gel, the amplified products were examined. By evaluating the gel's band quality, optimized annealing temperature was determined.

Expression Analysis

The study used cDNA samples from two cell lines that were treated with three different riproximin doses to perform qRT-PCR for ten genes. 2-ΔΔCT approach was used to determine gene expression changes in control and riproximin-treated samples. HPRT1 was used as a reference gene. 2-ΔΔCT (Livak) method was used to calculate fold changes from data of treated and untreated groups.

Table 1: Primer sequences of genes.

Genes	Primer Sequence (F)	Primer Sequence (R)
CASP9	GGAAGAGCTGCAGGTGGAC	CCTGCCCGCTGGATGTC
CCND1	GGGGGCGTAGCATCATAGTA	GTGGTGGCACGTAAGACACA
CD14	GAAGACTTATCGACCATGGAGC	AGACGCAGCGGAAATCTTCA
CDKN1B	CCGGCTAACTCTGAGGACAC	TGCAGGTCGCTTCCTTATTC
FASLG	CTGGGGATGTTTCAGCTCTC	CTTCACTCCAGAAAGCAGGAC
FOS	GGGGCAAGGTGGAACAGTTA	AGGTTGGCAATCTCGGTCTG
JUN	ACGGCGGTAAAGACCAGAAG	CTCGCCCAAGTTCAACAACC
NFKB1	CCTACGATGGAACCACACCC	ATCTGCTCCTGCTGCTTTGA
PDK1	TCCTGGACTTCGGATCAGTG	TGCAACCATGTTCTTCTAGGC
PIK3R2	CAGAGAGATCGACAAGCGCA	GTGCGTACTGGTCCTCAGTC
GAPDH	ACGGATTTGGTCGTATTGGG	CGCTCCTGGAAGATGGTGAT
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

RNA and cDNA Verification

Total RNA was extracted from untreated and treated breast cancer cell lines using a commercial extraction kit, revealing high purity (260/280 ratio: 1.8-2.0) and quantity (>50ng/µl/sample). cDNA was synthesized using extracted total RNA and verified by using PCR-based amplification of a reference gene (GAPDH). Agarose gel electrophoresis was used to visualize the amplified product, revealing an intact, high-quality synthesis of cDNA from the extracted RNA samples, shown in Figure 1.

Primer Optimization

Primers for ten selected genes were designed and optimized by using gradient PCR methodology and visualized on agarose gel electrophoresis, indicating good quality in design and optimization protocols. Based on intensity of bands, appropriate amplification conditions were selected for subsequent experiments. Gel electrophoresis pictures of the optimized genes are shown in Figures 2-4.

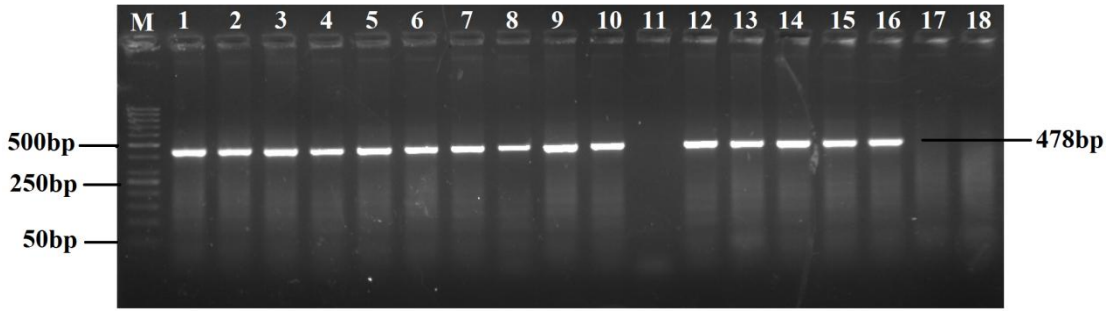

Figure 1: Electrophoresed cDNA of MDA-MB-231 and MCF-7; Sample distribution (left to right): Well 1: DNA marker (50bp) Well 2-9: MDA-MB-231 (untreated and treated samples in duplicate), Well 10-16: MCF-7 (untreated and treated samples in duplicate), Well 17-18: PCR negative controls.

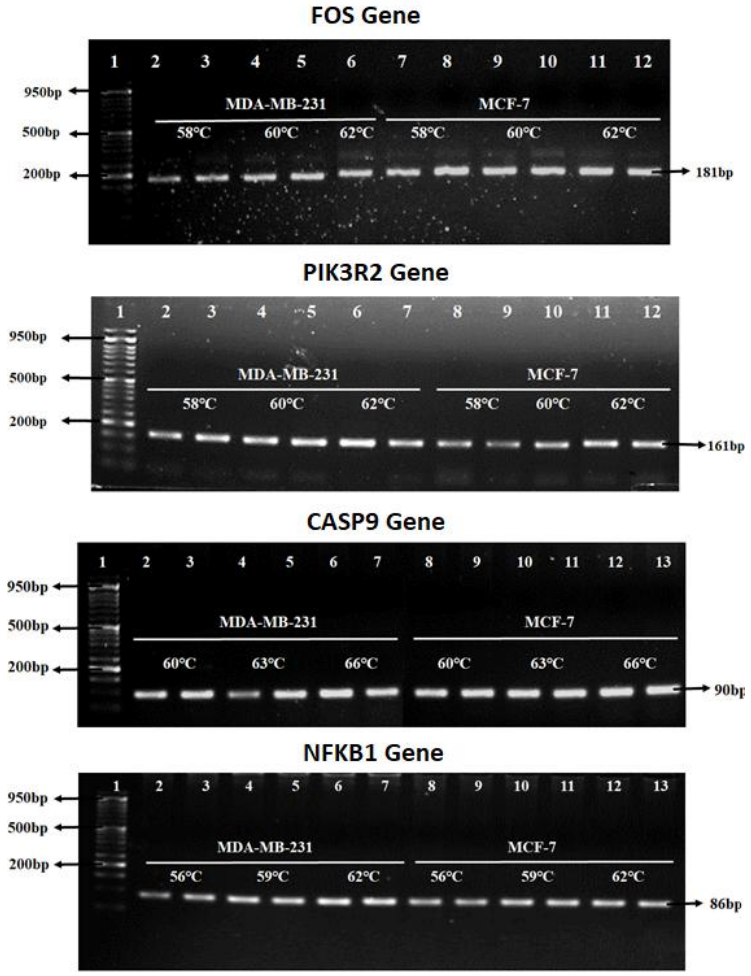

Figure 2: Primers showed specific amplification at different annealing temperatures for FOS, PIK3R2, CASP9 and NFKB1 genes. Sample distribution (left to right), well 1: DNA marker (50bp), well 2-13: MDA-MB-231 and MCF-7 untreated cell lines. Amplified products were visualized on 2.5% agarose gel electrophoresis.

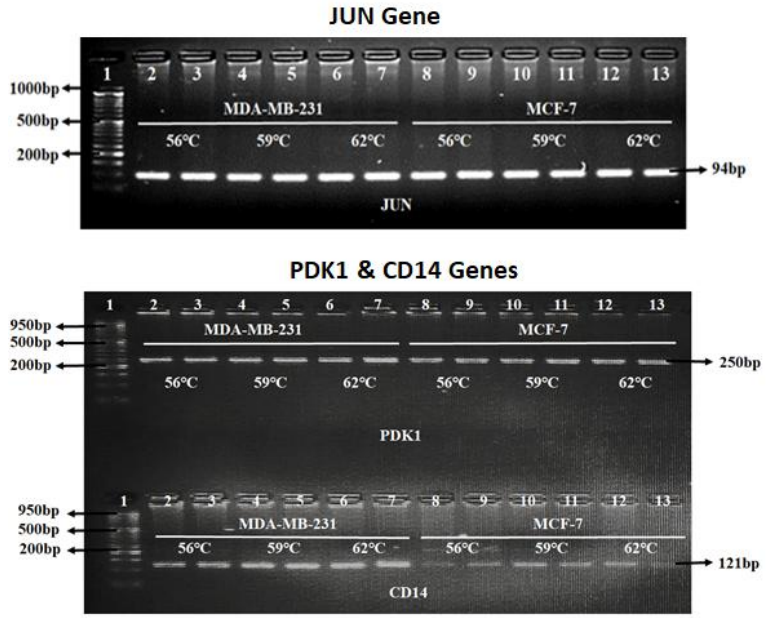

Figure 3: Primers showed specific amplification at different annealing temperatures for JUN, PDK1 and CD14 genes. Sample distribution (left to right), well 1: DNA marker (50bp), well 2-13: MDA-MB-231 and MCF-7 untreated cell lines. Amplified products were visualized on 2.5% agarose gel electrophoresis.

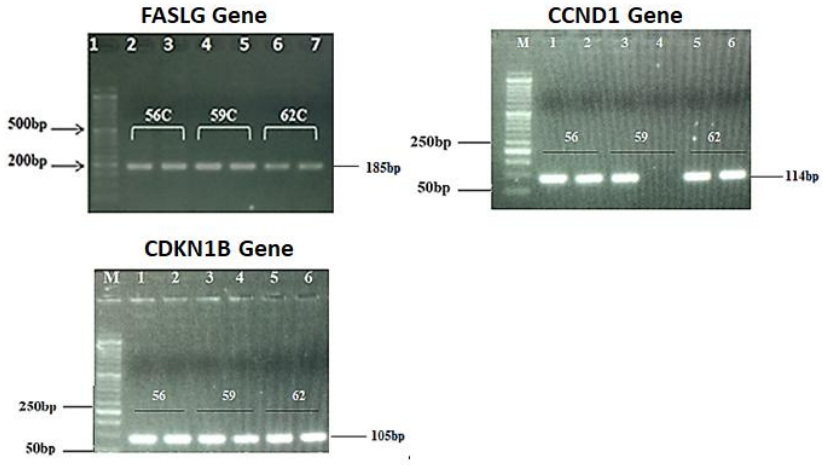

Figure 4: Primers showed specific amplification at different annealing temperatures for FASLG, CCND1 and CDKN1B genes. Sample distribution (left to right), well 1: DNA marker (50bp), well 2-13: MDA-MB-231 and MCF-7 untreated cell lines. Amplified products were visualized on 2.5% agarose gel electrophoresis.

Expressional Analysis

In this study, we evaluated the effects of riproximin on expression levels of the selected ten genes in breast cancer cell lines after exposure to three different concentrations. Expressional analysis was performed using qRT-PCR, identifying expressional modulations in MDA-MB-231 and MCF-7 cell lines, shown in Figure 5 (MDA-MB-231) and Figure 6 (MCF-7). Only three genes (CD14, CDKN1B, and JUN) in MDA-MB-231 cell line and two genes (CASP9 and JUN) in MCF-7 cells showed concentration-dependent effects. As far as the maximum de-regulation is concerned, FOS gene was maximally up-regulated (36fold) followed by NFKB1 (20fold) and Jun (18fold) in MDA-MB-231 cells. In MCF-7 cells, most effective de-regulation was observed in CD14 (19fold) followed by NFKB1 (12fold). More interestingly, these maximum de-regulations in MCF-7 cells were observed in response to lowest concentrations of riproximin (1ng/ml). All in all, riproximin showed a prominent potential to alter the expression of PIK3-AKT-mTOR pathway genes in cell specific manner.

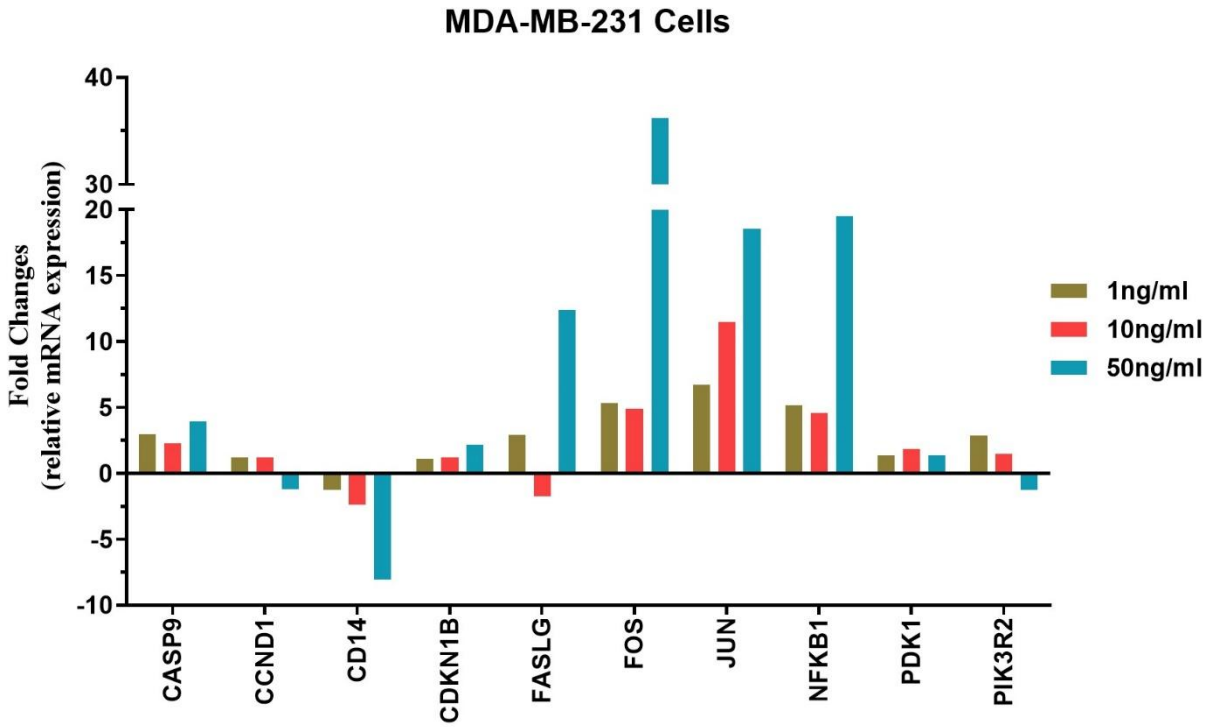

Figure 5: Fold changes in selected genes in MDA-MB-231 cell line. The cells were exposed with three different concentrations of riproximin followed by qRT-PCR based analysis. Fold changes were determined by using Livak method.

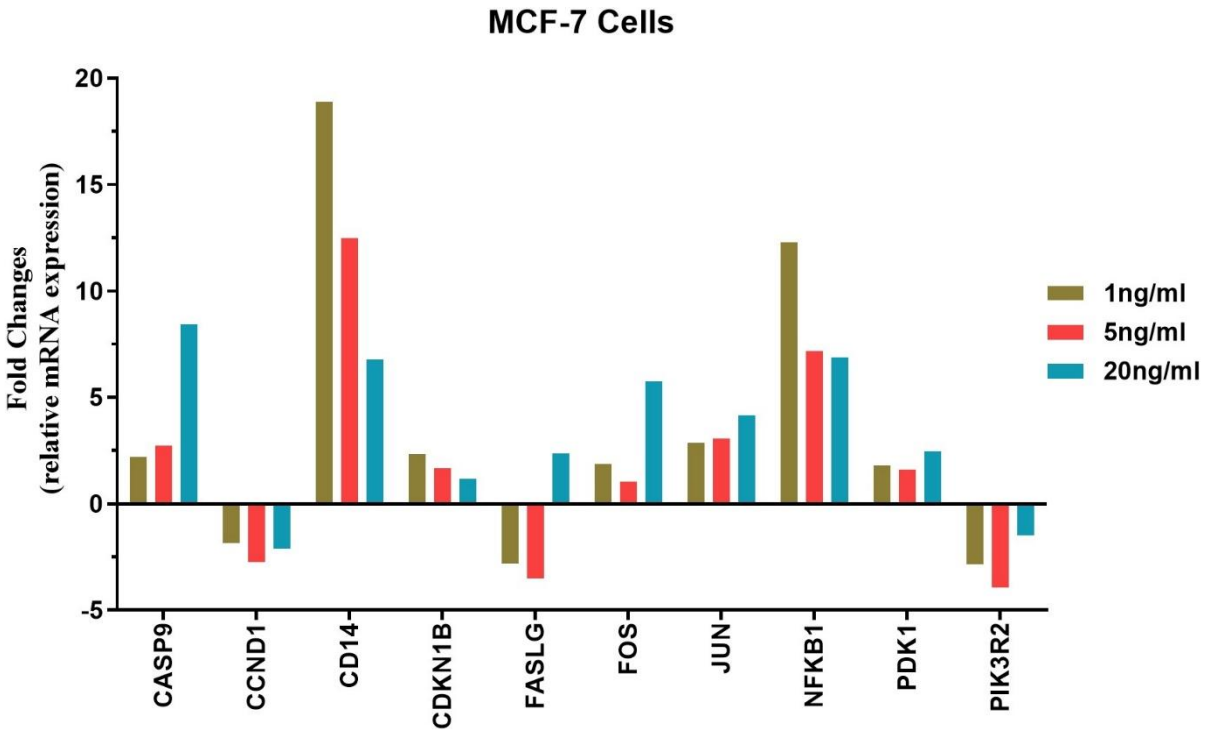

Figure 6: Fold changes in selected genes in MCF-7 cell line. The cells were exposed with three different concentrations of riproximin followed by qRT-PCR based analysis. Fold changes were determined by using Livak method.

DISCUSSION

Indeed, the leading cause of morbidity and death worldwide is breast cancer. When combining the cases, the incidence rate is rising quickly and overtaking lung cancer [16,17]. A major hurdle in controlling breast cancer is heterogeneous nature of this disease, aggressive behavior and lack of effective therapeutic options [18]. In this context, it is of paramount importance for the scientific community to search for new safer but effective treatment options. Plants can be an attractive source of medical entities in this scenario. A huge variety of plants have been studied so far to explore anticancer components from their different parts including roots, leaves, and fruits [19]. *X. americana* is one kind of such plant, which has been used for its medicinal benefits in African countries. Particularly, seeds of this plant (in grounded form) have been used by local people to treat various diseases including cancers [20].

Several fractions from *X. americana* seeds were isolated in 2006, and their corresponding anticancer properties were examined [21]. Riproximin, a highly effective antineoplastic protein from this plant, has been investigated over time for its ability to combat a variety of malignant cell lines and animal models [22]. There have been several demonstrated genetic changes brought about by riproximin in these cell lines; nevertheless, information regarding the precise effects of this protein on different pathways is scarce. It is necessary to clearly emphasize riproximin's effects at the molecular level in order to comprehend the changes it is imposing on different signaling pathways. With that in mind, the PI3K-AKT-mTOR signaling cascade one of the most important and dysregulated cascades in malignant cells was the subject of our discussion.

Core part of this study was to identify the impact of riproximin exposure on PI3K-AKT-mTOR signaling related genes in the breast cancer cell lines. For this purpose, the cells were exposed to different concentrations (MCF-7: 1-20ng/ml, MDA-MB-231: 1-50ng/ml) for 48 hours followed by RNA extraction, cDNA synthesis, and real-time PCR based amplification. The treatment concentrations were selected based on a previous MTT toxicity data sets, where the aim was to use higher concentrations for having sufficient effects while avoiding too much cellular death to have enough cells for extraction of RNA at the same time [15]. Overall, the data generated from these results showed that MCF-7 cells were more responsive towards riproximin as compared to MDA-MB-231 cancerous cells. Based on known literature and a reference from Qiagen RT^2 PCR panels, a total of 10 genes related to PIK3-AKT-mTOR pathway were selected to be examined in this study. Real-time PCR data showed a discrete pattern of alterations in the expressions in response to riproximin exposure. Most effectively up-regulated genes in MDA-MB-231 cells were FOS (36fold)

followed by NFKB1 (20fold) and JUN (18fold). Interestingly, all these maximum up-regulations were observed in response to the highest applied concentrations (50ng/ml) in MDA-MB-231 cells, which shows that the cells responded in a linear fashion towards the tested compound. In contrast, a different set of de-regulation was observed in MCF-7 cells, where CD14 (19fold) was the most affectively de-regulated gene followed by NFKB1 (12fold). More importantly, these extreme de-regulations were observed in response to the lowest applied concentration (1ng/ml) in MCF-7 cells, which in turn shows the development of resistance or irresponsiveness of the cells towards higher concentrations of riproximin in MCF-7 cells. Another interesting fact is the existence of a differential response in de-regulations: for instance, CD14 was effectively inhibited in MDA-MB-231 cells (-7fold) while it was induced in MCF-7 cells (19fold). This reflects that cancer cell types from the same malignancy can respond differently towards riproximin exposure, like MDA-MB-231 triple negative cells (ER^-/PR^-)/$HER2^-$) did as compared to MCF-7 double positive cells (ER^+/PR^+). Nevertheless, riproximin exhibited a substantial potential to de-regulate PIK3-AKT-mTOR pathway related genes in breast cancer cells and needs further investigations to target this pathway for therapeutic purposes. Provided with further *in vitro* and *in vivo* investigations, the compound can be an affective regulator of this vital proliferation related pathway in cancer cells.

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Contributions: AK and KU perfomed experiments. OS analyzed data. AP planned the work and drafted the manuscript.
Competing Interests: No competing interests
Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.