Suppression of NF-κB and Activation of Fas/CD95 Apoptotic Pathways by Riproximin in MDA-MB-231 and SW-707 Cancer Cells

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Abstract

Riproximin (Rpx), a type II ribosome-inactivating protein has high specificity for cancer glycans. The present study investigates the capacity of Rpx to engage the CD 95/FAS death receptor in human breast (MDA-MB-231) and colorectal (SW-707) cancer cells lines. To investigate the effect on apoptotic genes whenexposingMDA-MB-231 cells to Rpxat IC_{50}concentration,a cDNA microarray was performed. Interestingly, Rpxtreatment caused a significant reduction ($p \leq 0.05$) of *IKBKB*, *NIK, BCL2* and *ROCK1*mRNAexpression,whereas a significant upregulation of *BAX*, *CASP3*, *CASP9*, *BID* and *ACIN1*mRNA expression was induced. At protein level, a significant reduction of NF-κB in both treated cell lines was observed. Additionally, anti-apoptotic proteins were either not affected or were significantly reduced whereas pro-apoptotic proteins showed significantly increased expression. Of importance, Rpx was found to directly bind to the FAS death receptor activating both, the extrinsic and intrinsic apoptosis pathways through engagement of the FAS Associated Death Domain (FADD) and subsequent activation of the caspase cascade. These findings indicate that Rpx suppresses cancer cell proliferation and survival by inactivating NF-κB and ligating FAS to cause apoptosis in both cancer cell lines and point to the potential application of this lectin as a targeted anti-tumor agent.

Introduction

Cancer is currently the second leading cause of death worldwide and is expected to further increase in importance within the 21st century [1]. Worldwide cancer statistics reported a rise to as many as 18.1 million new cases and 9.6 million cancer deaths in 2018. From these, in terms of new cases, breast cancer accounts for 11.6% and colorectal cancer for 10.2% [1]. Current traditional treatment options for cancer are associated with high toxicity, resistance and relapse rates, are expensive [2];[3] and therefore, there is a need to develop new, safe and more effective anti-cancer agents. Furthermore, the current antineoplastic compounds are associated with toxicity to both, the aberrant as well as the normal body cells. Part of this toxicity is caused through activation of the apoptotic machinery. Apoptosis, the cells' natural mechanism for programmed cell death, is important for both development and homeostasis and is particularly an important process in metazoans. Following an external or internal signal for apoptosis, caspases are activated leading to cleavage of cellular components resulting in cell apoptosis [4]. Apoptosis in cancer cells is an important area of investigation with respect to the development and formulation of anti-cancer compounds. The apoptotic pathway involves activation by either intracellular or intrinsic signals recruiting the mitochondria, or extracellular or extrinsic signals recruiting death receptors. Most conventional anti-cancer therapies have been reported to develop resistance to the intrinsic pathway of apoptosis in cancer cells [3]. In addition, these first-line cancer therapies are associated with high toxicity rates and hence, there is a need to search for new safe and effective anti-cancer treatments.

A promising option within the search for new anticancer agents is the identification and characterization of plant based natural products.

Riproximin is a 58-62 kDa lectin and is the active ingredient of *Ximeniaamericana,* a widely distributed small tree in tropical and subtropical regions [5];[4]. The anti-neoplastic activity as well as the safety of Rpx has already been established in previous studies. Rpx was shown to have a highly selective toxicity against cancer cell lines and at effective concentrations showed no effect or onlyminimal poisonousness to normal cell lines [6];[7]. Rpx is a type II ribosome-inactivating protein (RIP) and like other RIPs, has two polypeptide chains, A and B, linked to each other

by a disulfide bond. The A chain is an rRNA*N*-glycosidase that causes depurination of the 28S rRNA,thus resulting in transcriptional arrest by blocking protein synthesis [4]. The B chain binds to cell surface glycans, thus initiating the internalization process of the lectin. A high affinity of Rpx for cancer cell surface structures including bi- and tri-antennary N-glycans and O-glycans has been reported[8]. The internalization of Rpx is followed by cell death through apoptosis. Thus, Rpx is a potent anti-cancer agent and may be involved in the killing of tumor cells bymore diverse apoptotic mechanisms than previously described. Previous studies reported Rpx as an effective anti-cancer agent the activity of which depends on gene expression that in turn sensitizes pancreatic ductal adenocarcinoma (PDAC) cells to TRAIL [9]. In addition, Rpx has been shown to induce IL24/MDA-7 and GADD genes in colorectal cancer cell lines [4]. Another study conducted in our laboratory reported the ability of Rpx to induce the unfolded protein response in human cancer cells including MDA-MB-231 and HCT 116 [10]. Thus, Rpx has a wide span of anti-tumor activities and further research may reveal more interesting mechanisms of action of this valuable natural anti-cancer agent. The effectiveness of Rpx as a natural-product-based anti-tumor agent is maybe more attractive than that of many other plant-based cancer therapies. For example, treatment of colorectal cancer cell lines, SW480, SW620 or CC531 with Rpx reported the highest 48 h IC_{50} to be 2.1 ng/ml [4], a value which is about 1,000 times lower than respective IC_{50}values obtained from plant extracts. Such extracts typically range in the order of 1 -100 μg/ml,as reported recently for the acetone extract of the bark of *Canariumodontophyllum* in the treatment of HCT116 human colorectal cancer cells[11].

Effective anti-cancer agents may be involved in interfering with the machinery causing increased cell proliferation and survival or may act as ligands for death receptors, in both cases, triggering cell apoptosis. NF-κB regulates genes involved in cell proliferation and survival, and shows common dysregulation of its activity in cancer [12].

When dysregulated, the transcription factor maintains its active form and turns on the expression of genes associated with cell proliferation and survival, thus protecting cancer cells from death that may result from apoptotic stimuli.

This sustained cell proliferation is reflected in metastasis and also in tumor host immune evasion [12]. Since NF-κB is active in tumor cells [13], suppression of this transcription factor will reduce cancer cell proliferation and causecell death. Thus, research strategies aimed at inhibiting the signaling of NF-κB, have potential anti-cancer therapeutic applications [14];[15];[16]. Such a therapeutic approach must also be able to suppress NIBP [NIK (NF-kappa-B-inducing kinase)and IKBKB(Inhibitor of nuclear factor kappa-B kinase subunit beta)-binding protein], which is associated with activation of NF-κB. NIBP is a key regulator of the NF-κB signaling pathway and has been associated with colorectal cancer metastasis [17]. The knockdown of NIBP suppresses CRC metastasis through downregulation of NF-κB and this highlights the importance of a cancer therapeutic approach, which blocks the role of NIBP in cancer cells. Death receptors such as Fas/CD95 are members of the tumor necrosis receptor (TNF-R) superfamily. As members of this family do not have intrinsic enzymatic activity, they rely on dynamic formation of protein complexes to network with enzymes and signals, and this feature contributes to the versatility of the receptor family members in activating signaling pathways. It has been hypothesized that activation or inhibition of the CD95 receptor may be an attractive therapeutic approach for cancer patients [18]. The FasL will activate CD95 to trigger apoptosis in cells. On the other hand, a therapeutic approach inhibiting CD95 may as well trigger the receptor to activate the apoptotic process in normal cells. For example, a monoclonal antibody against CD95/Fas/apoptosis antigen 1, is able to trigger cell death in tumor cells by targeting this receptor. Thus, some therapeutic agents may activate or inhibit CD95 and in both cases lead to apoptosis in targeted tumor cells. CD95 transmits death signals through the adaptor protein, FADD (Fas associated death domain), that triggers the activation of the caspase cascade leading to apoptosis in target cells. Upon receiving death signals from a death receptor, FADD engages caspase-8 through an element known as death effector domain, present in both FADD and in the prodomain of the caspase. Caspase-8 plays a central role in initiating apoptosis upon activation of death receptors by extrinsic factors [19]. Cleaved caspase-8 activates caspase-3 leading to its cleavage and the cleaved form will execute apoptosis in cells by the extrinsic pathway. At the same time, cleaved caspase-8 may activate BID leading to its cleavage into truncated BID (tBID), which then activates BAX on the surface of mitochondria leading to permeabilization of the mitochondrial outer membrane[20]. This leads to release of cytochrome Cfrom the mitochondria. Cytochrome Cactivates the apoptosome, the complex between ApaF-1 and caspase-9, leading to cleavage of caspase-9 [21]. Cleaved caspase-9 activates also caspase-3, leading to its cleavage to execute apoptosis in cells by the intrinsic pathway. Cleaved caspase-3 may also cleave Acinus (apoptotic chromatin condensation inducer 1, coded by the ACIN1 gene) leading to apoptotic chromatin condensation resulting in death of treated cells [22]. Another important gene located downstream to caspase-3 that may be important as a therapeutic target in tumors is ROCK1, which is involved in cell shrinkage and membrane blebbing. Increased phosphorylation of myosin light chain (MLC) is

central in function to the dynamic membrane blebbing observed at the onset of apoptosis. ROCK1, an effector of the small GTPase Rho, acts as a substrate for caspases. ROCK1 is cleaved by activated caspase-3 at a conserved sequence resulting in removal of its inhibitory carboxy-terminal domain. Cleaved ROCK proteins in apoptotic cells lead to increased levels of phosphorylated MLC [23]. Because ROCK functions as a key modulator of cell motility, angiogenesis, tumor cell invasion and metastasis [24];[25], its inhibition by anti-cancer agents or its cleavage by activated caspase-3 is an important tactic in cancer treatment.

Therapies targeting the CD95 or its ligand, or interacting with the death receptor resulting in apoptosis in cancer cells, may be an attractive option to the treatment of cancer. An exceptionally effective anti-cancer therapy would be able to trigger both, the extrinsic and intrinsic pathways of apoptosis. This can, to a large extent, ensure therapeutic potency with minimal or no chances of the cancer cells developing resistance to treatment. Effective therapies will be those that inhibit anti-apoptotic components and their mechanisms while at the same time activating the pro-apoptotic components. Important anti-tumor drugs would inhibit members of the BCL2 family of proteins including Bcl2, which is involved in suppressing BAX to achieve cell survival and progressive growth. Suppression ofBCL2 and up regulation ofBAX would lead to successful inhibition and death of tumor cells [3].

While reports indicate that Rpx can inhibit the growth of various types of cancer cells, and that it is highly safe, with lownoxiousness to normal cells but with high specific toxicity against tumor cells, there has been no attempt to investigate the effects of Rpx treatment on NF-kB or cell surface death receptors and the associated apoptosis pathways in cancer cells. Motivated by the unique nature of Rpx in interacting and binding to receptors or aberrantly expressed antigens on the surface of tumor cells, this study sought to investigate whether Rpx could bind to Fas/CD95 death receptor and cause apoptosis in MDA-MB-231 breast and SW-707 colorectal cancer cell lines. The effect of treating cells with Rpx on FAS, FASL, BAX, BAK, CASP9, CASP8, CASP3, BCL2, BID, ROCK1, IKBKB, NIK and ACIN1 genes and/or protein expression and activation status was investigated. The effect of treatment of the selected cancer cell lines with riproximin on NF-κB protein expression level was also determined.

Materials and methods

Cell lines and culture conditions

Human MDA-MB-231 breast and SW-707 colorectal cancer cell lines were cultured in RPMI-1640 medium (Invitrogen, Darmstadt, Germany) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). The cell lines were maintained under sterile standard incubation conditions with a humidified atmosphere (37°C,5% CO_2). Cells were passaged at appropriate intervals to maintain logarithmically growing populations.

Reagents and antibodies

The identification, characterization and purification of Rpx have been clearly described [7, 8,26]. MTT was obtained from SERVA Electrophoresis (Heidelberg, Germany), culture media and L-glutamine was purchased from Invitrogen (Darmstadt, Germany), and FBS from PAN Biotech. Protease-inhibitor-cocktail Complete Mini was sourced from Roche Applied Science (Penzberg, Germany). Primary antibodies against cytochrome C (D18C7), cleaved caspase-3 (Asp175) (5A1E), Phospho-NF-$_k$B p65 (ser536) (93H1), cleaved caspase-8 (Asp391) (18C8), caspase-9 (Human specific), Fas (C18C12), FasL (D1N5E), BID (Human Specific), Bax (D2E11), and Bcl-2 (Human Specific) were purchased from Cell Signaling Technology (Danvers, MA). FADD (H-10): sc-271520, Bak (N-20): sc-1035 and GAPDH (0411): sc-47724 primary antibodies and the secondary antibodies, goat ant-rabbit IgG-HRP (sc-2030) and goat anti-mouse IgG-HRP (sc-2005) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The human soluble Fas (sFas; C103, Cat 310-20) receptor protein was purchased from PeproTech EL Ltd (London, UK).

Cell viability

MTT assays were performed to determine the IC_{50} of Rpx for the selected cancer cell lines. Cells were seeded at a density of 5 x 10^3 cells / 100 µL of culture medium per well in 96-well plates and incubated at 37°C in 5% CO_2 under humidified conditions. After 24 h of incubation, Rpx was added to wells in complete cell culture medium in serial dilutions (0.1953125-100 ng/ml) and plates were further incubated for 48 h. MTT solution was added to each well (20 µl of 0.5 mg/ml of MTT) and incubated for 3 h at 37°C before formazan crystals were solubilized in isopropanol-HCL solvent. The absorbance of the dye was measured spectrophotometrically at 540 nm and 690 nm

(reference) using an automated microplate reader (AnthosMikrosysteme, Krefeld, Germany). The percentage of cell growth inhibition was calculated by comparing the mean absorbance values of treated cells with that of untreated cells. Reported results are the mean values of MTT assays performed in triplicate.

Protein isolation and western blot analysis
The cells were exposed to Rpx (IC_{50}) for 48 h and whole cell lysates were prepared from both treated and untreated cells using RIPA buffer plus, Sodium vanadate, phosphatase and protease inhibitors at 4°C for 30 minutes with constant shaking. The lysates were centrifuged (14,000xg at 4°C) for 20 minutes and the supernatants were immediately transferred to fresh tubes and stored at -20°C until analyzed for protein levels. Protein concentration was determined using the Roti-Nanoquant reagent (Carl Roth GmbH+Co. kG, Germany). For Rpx-protein (pure) or Rpx-cell lysate complex formation analysis, Rpx (5 µg per well) was run through a gel by electrophoresis before transfer onto a PVDF transfer membrane (ref 88520; Thermo Scientific, Germany). Following blocking with 5% blocking solution, the membrane was incubated in a solution containing the target protein or lysate overnight followed by washing before another overnight incubation with the specific primary antibody. After a brief washing step, membranes were incubated with HRP-conjugated secondary antibody for 1 h before addition of substrate and reading of results.

Microarray analysis
MDA-MB-231 cells were treated with Rpx IC_{50} for 48 h and RNA was extracted from the cells with RNeasy Mini Kit (Qiagen, Hilden, Germany). Extracted RNA quality was determined by gel analysis and RNA Nano chip assay using am Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). RNA samples with sufficient quality (RNA integrity number (RIN) values ≥8.8) were selected for gene expression analysis.

Statistical analysis
Data were analyzed using GraphPadInStat software and suitable statistics were applied accordingly. One way analysis of variance was used to analyze mean values of western blot protein levels. ImageJ and GIMP 2 software programswere utilized in analyzing images from western blot assays. A *P* value ≤0.05 was considered statistically significant.

Results and discussion

Rpx inhibits growth of breast and colorectal cancer cells *in vitro*
Following exposureof human MDA-MB-231 breast or SW-707 colorectal cancer cells to increasing concentrations (0.195 to 100 ng/ml) of Rpx for 48 h, the anti-proliferative activity against these cells was measured by MTT assay. The results indicatedan IC_{50} of 2.17 ± 0.02 ng/ml for the breast cancer and of 3.125 ± 0.03 ng/ml for the colorectal cancer cells. Both cell lines showed a concentration-dependent sensitivity to Rpx,with a tendency to a stronger growth inhibition in MDA-MB-231 than in SW-707 cells. The increasing level of cell growth inhibition with increasing concentration of Rpx, indicating early cytotoxicity at low concentration of the test compound, is a confirmation of the potency of this compound in treating cancers of various origin as described before[4];[7]. Furthermore, the low IC50 values for the two cell lines arein line with the fact that Rpx is a pure compound and differs in its highly specific activity from that of extracts [6, 7]. Previous findings in our laboratory had reported high antiproliferative efficacy of Rpx against rat CC531[7] and human HCT116 [10], SW 480 and SW 620 [4]colorectal cancer cells, among other studies. The small difference in IC_{50} values reported for SW 480 or SW 620 cells [4]as well as the $IC_{50}$for SW-707 cells reported in the current study indicate a similarly high activity in various colorectal cancer cell lines and thus suggest colorectal cancer as a potential target of Rpx.

In addition to colorectal cancer cells, Rpx has been shown to be highly effective in cancer cells derived from other tissues as well, including breast, liver, brain, lung, bone, and bone marrow [7]. Comparing the efficacy of extracts with that of Rpx as a pure compound, there is a 1000 fold lower specific activity of the former as compared to the latter [6];[7]. This observation is paralleled by a recent study using extracts from *Canariumodontophyllum* stem, which reported an IC_{50} value of 82 µg/ml against HCT116 colorectal cancer cells [11].

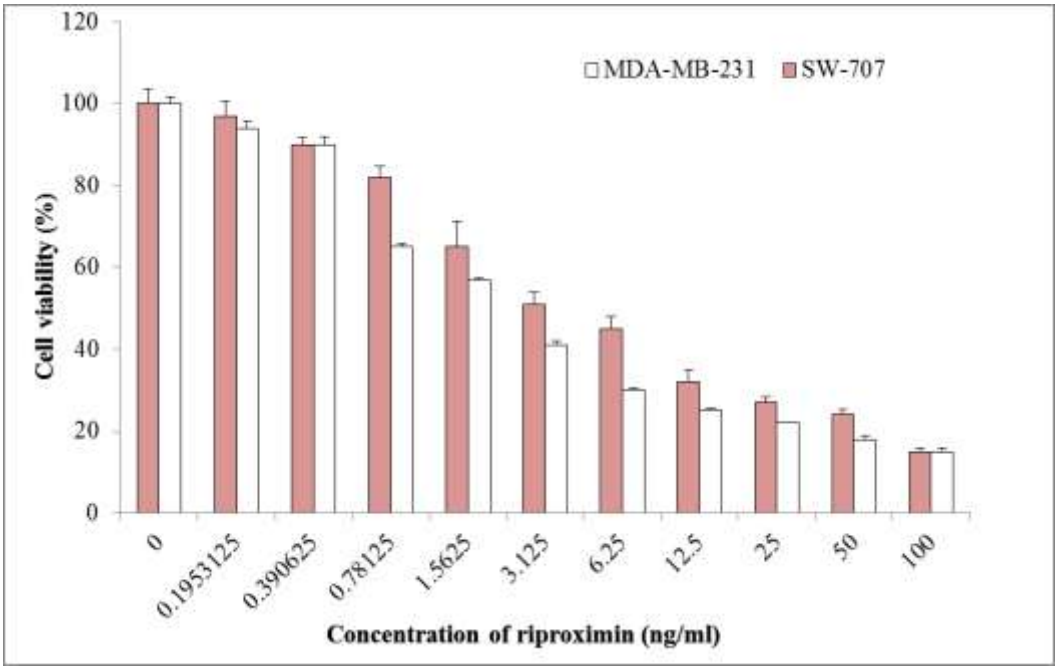

Figure 1: In vitro cytotoxic effects of riproximin (Rpx) in human MDA-MB-231 breast and SW-707 colorectal cancer cells. Cancer cells were treated with increasing Rpx concentrations for 48 h and cell viability was assessed by MTT assay.

Treatment of MDA-MB-231 cells changes the transcriptionlevel of key genes involved in apoptosis

To unravel the effects caused by Rpx on apoptotic genes, a microarray analysis of RNA from both, treated and untreated MDA-MB-231 cells was carried out and results are illustrated in Figure 2. Following Rpx treatment of MDA-MB-231 cancer cells, mRNA transcription increased significantly for *BID*, *BAX*, *CASP9* and *CASP3* as well as the apoptotic chromatin condensation inducer (*ACIN1*) gene, whereas*BCL2* that promotes cellular survival and inhibits the actions of pro-apoptotic proteins, recorded a significantly decreased transcription ($p \leq 0.05$). Expression levels of *IKBKB* and *NIK,* which are both involved in the activation of NF-$_k$B, were significantly reduced. The *ROCK1* gene, involved in cell motility, tumor cell invasion, metastasis and angiogenesis was substantially suppressed recording a significant reduction when compared to the untreated control. However, treatment of MDA-MB-231 cells with Rpx hadno significant effect on mRNA expression levels of *CASP8*.

The upregulation of important apoptosis components including *ROCK1*, *ACIN1*, *caspases*, and *NF-$_k$B* in response to Rpx treatment of tumor cells is a new addition to the search for effective anti-cancer agents. ROCK1 plays a role in cancer development and is particularly associated with metastasis and plasticity as well as angiogenesis [25] and disease severity. Thus, the significant reduction in ROCK1expression as a result of its activation through cleavage by caspase-3 in Rpx-treated MDA-MB-231 is a confirmation of the effectiveness attributable to this anti-cancer agent. Another rarely researched, but important potential anti-cancer target is *ACIN1,* which leads to chromatin condensation following activation by cleavedcaspase-3 during cell apoptosis [22]. The significant upregulation of *ACIN1* in Rpx-treated MDA-MB-231 cancer cells indicates an important featureof Rpx i.e. itstargeting of key apoptotic pathways in tumors with consequences of cell death.

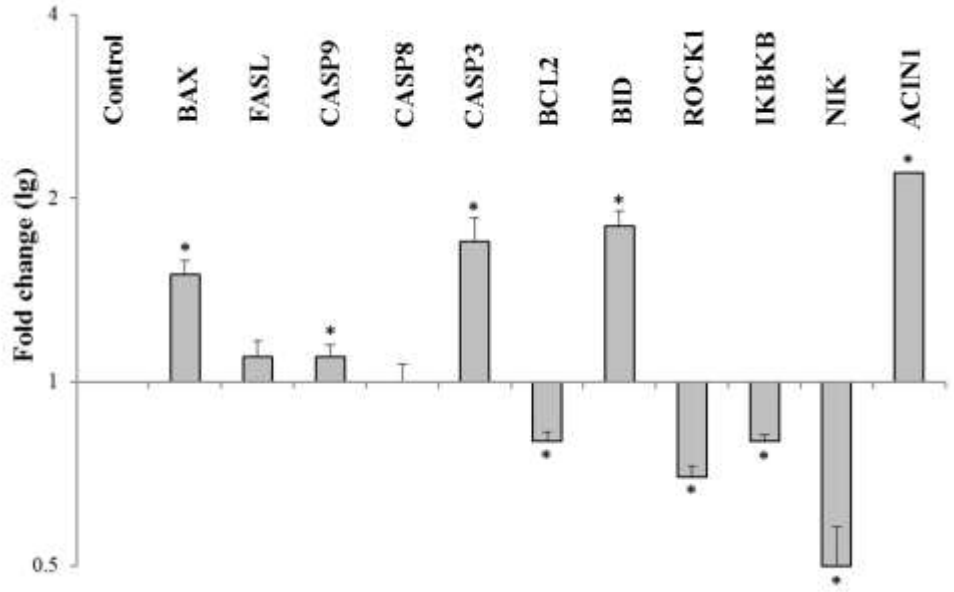

Figure 2: Modulation of selected genes (BAX, CASP9, CASP8, CASP3, BCL2, BID, ROCK1, IKBKB, NIK and ACIN1) in human MDA-MB-231 breast cancer cells following 48 h exposure to$IC_{50}$of Rpx. The y-axis (lg scale) gives the fold change in gene expression as determined by microarray analysis comparing control and Rpx exposed MDA-MB-231 cells. **Asterisks indicate a significant difference (p≤0.05) as compared to the non-treated control.**

Treatment with Rpx suppresses NF-κB protein expression

Treatment of human MDA-MB-231 or SW-707 cancer cells with corresponding IC_{50} values of Rpx for 48 h was associated with a significant (p≤0.05) reduction of NF-κB protein expression in both treated cell lines as compared to untreated cells. A reduction by 56% and 66% was achieved in MDA-MB-231 and SW-707 cells, respectively (Figure 3). In tumors, activated NF-κB leads to increased expression of genes, which promote cell plasticity with associated increased cell division, proliferation, metastasis and severe disease progression [27]. An effective strategy to inhibit tumor growth would be to inhibit the aberrantly expressed pro-survival components such as NF-kB and its associated activating elements. Findings from the present study clearly indicate that Rpx is an effective anti-cancer agent given its potent capacity to significantly inhibit the expression of NF-kB in both, MDA-MB-231 and SW-707 cancer cells. Interestingly, a significant reduction in the expression of NIBP in Rpx-treated MDA-MB-231cancer cells was observed using the microarray analysis. NIBP binds directly with NF-kB leading to activation of the latter [28] and therefore, disruption of the components of NIBP (NIK and / or IKBKB) with an anti-cancer agent would lead to apoptosis by suppressing NF-kB and its pro-survival activities in tumor cells, as observed in the present findings. This desirable effect of Rpx i.e. inhibition of NF-kB and NIBP, may be an indicator that tumors treated with this agent may not easily become resistant to Rpx because of its feature of targeting more than one vital signaling chain.It appears also appropriate to state that-although Rpx treatment of both, breast and colorectal cancer cells, was significantly effective as measured by suppression of the NF-kB protein expression- this effect was more pronounced in SW-707 than in MDA-MB-231 cells. A recent study reported NF-kB as a potential therapeutic target in human colon cancer [27]. It has also been reported that knockdown of NIBP reduces colorectal cancer metastasis through down regulation of NF-kB [17].Our results indicate that such a knockdown can also be achieved by a suited drug, which in itself combinesvarious mechanisms for increasing the antineoplastic efficacy.

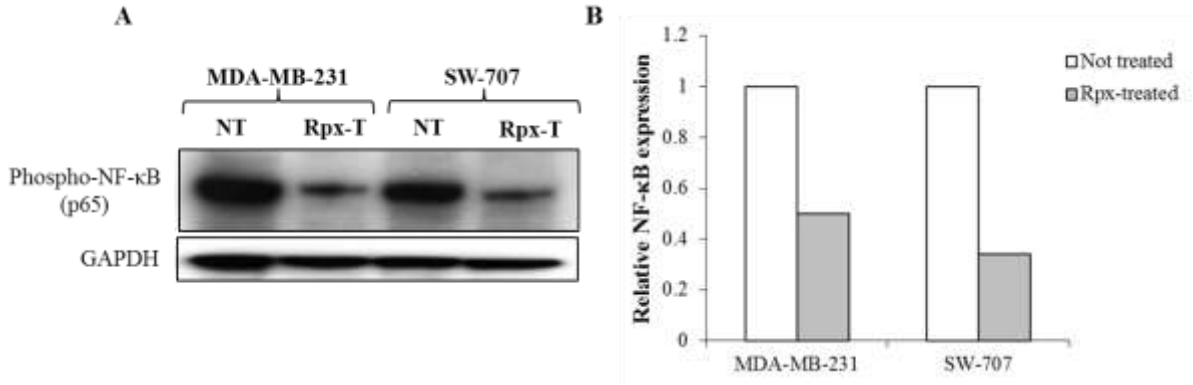

Figure 3: Protein expression of NF-κB in MDA-MB-231 and SW-707 cells following 48 h exposure to Rpx (IC_{50}) as determined by western blot analysis (A). Values in the graph are calculated relative to GAPDH expression as a loading control.

Rpx binds to human soluble Fas receptor

Exposure of MDA-MB-231 and SW-707 cancer cells to Rpx had no effect on the expression of Fas. Similarly, Rpx exposure changed the expression level of FasL non-significantly in both cell lines, with SW-707 cells showing a slight reduction and MDA-MB-231 cells showing mildly increased expression of FasL (Figure 4a,b).

Interestingly, Rpx was able to interact withpurified soluble Fas as shown from exposure of electrophoresed and blotted Rpx to purified soluble Fas (positive control) as well as to this protein contained in lysates from both cell lines (Figure 4c). In fact, antibodies detected soluble Fas bound to the specific bands of Rpx, i.e. the whole molecule, which ranges in size at 58-62 kDa, and tothe B chain, which ranges at 30-31 kDa.

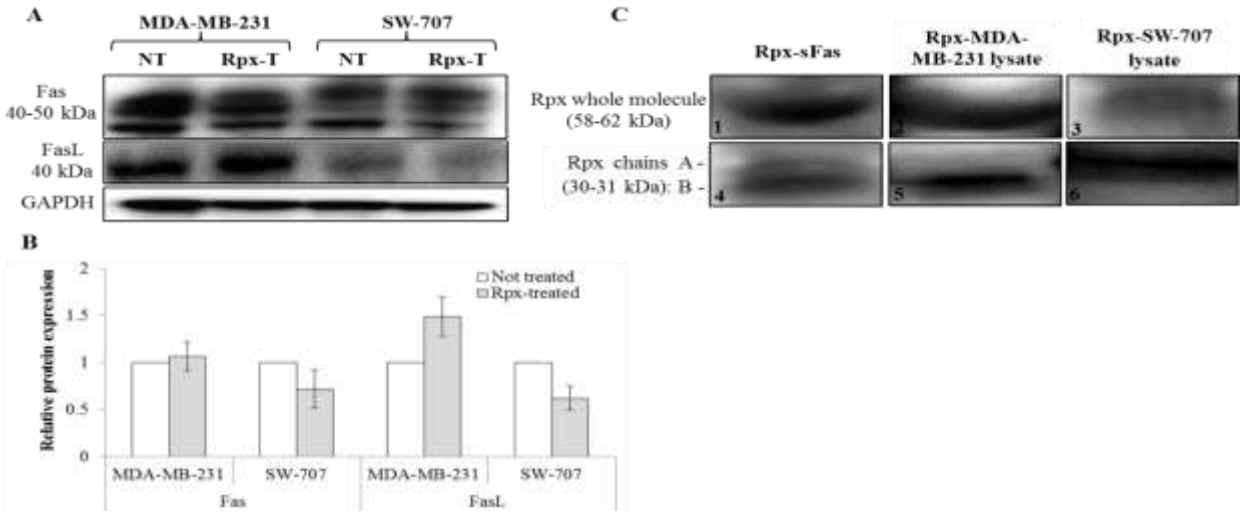

Figure 4: Protein expression of Fas and FasL in human MDA-MB-231 breast or SW-707 colorectal cancer cells and binding of riproximin (Rpx) with sFas or cell lysates. MDA-MB-231 and SW-707 cancer cells were treated with Rpx for 48 h and Fas or FasL protein expression was determined by western blot analysis (A) and expression levels were calculated relative to GAPDH expression as a loading control (B). Rpx was transferred onto a PVDF transfer membranein a western blot protocol and incubated with purified human soluble Fas protein or cell membrane proteins before detection with Fas specific antibodies. Rpx (under non-denaturing or denaturing conditions, thus leaving Rpx intact or separating it into its chains) formed complexes by bindingto purified soluble sFas protein (C1 and C4) or to the same protein contained in cell lysates from MDA-MB-231 (C2 and C5) or SW-707 cells (C3 and C6).

The demonstration that Rpx can directly bind to soluble Fas protein, which is present either in purified form or in cell lysates derived from MDA-MB-231 and SW-707 cells, is a key finding of the present study. Previous work on Rpx binding concentrated on Rpx-glycan complexes and glycan structures [4];[8]. The present findings leave open, whether Rpx binds to glycan structures of Fas or to the protein itself. This aspect will create a new area of interest

because it has not been reported so far that binding of molecules (including Rpx)to glycans of Fas can induce an engagement of the death receptor with its cytoplasmic death domain to trigger the activation of the caspase cascade. Such activation, however, has been demonstrated in this study (see below).

The Fas/CD95 receptor mediates activation-induced cell death following engagement with CD95L [29]. The complex CD95-CD95L (Fas-FasL) recruits an adaptor protein, FADD, which binds the death domain of caspase-8, leading to activation of the apoptotic effect or machinery. It has been well established that anti-cancer drugs may induce cell death through the CD95/Fas system and that a number of drugs commonly used in cancer chemotherapy induce CD95L/FasL expression in hepatocellular, neuroblastoma and leukemic cells leading to Fas-FasL engagement followed by cell death [30];[31];[32].

However, as indicated in the current results, Rpx treatment of MDA-MB-231 or SW-707 neither induced large production of FasL nor increased expression of Fas receptor in treated cells. Since apoptotic death occurred in both cell lines, and taking into account that this cell death was not caused by Fas-FasL engagement because their levels did not differ significantly from control, then it can be suggested that Rpx stimulated the death receptor, which then engaged a death domain with subsequent trigger of apoptosis. These observations are valuable attributes of an effective drug that utilizes the available death receptor repertoire to trigger significant apoptosis. In addition, and from a pharmacological point of view, causing an increased expression of CD95L may lead to unwanted side effects of the drug as the excessive CD95L may also engage with CD95 not only in tumor cells but also in healthy cells and trigger there undesired apoptosis.

Rpx causes activation of the caspase cascade and apoptosis in MDA-MB-231 and SW-707 cancer cells

Treatment of MDA-MB-231 or SW-707 cancer cells caused cleavage of the apoptosis initiator caspase-8 (Figure 5A). Caspase-9 (Figure 5B) and caspase-3 (Figure 5C) were also appropriately cleaved indicating activation of both, the extrinsic and intrinsic pathways of apoptosis. So far, this is the first study to determine the effects of Rpx on caspases and more so, on the caspase system involved in cancer cell apoptosis following treatment. Previous studies have reported that caspase-3, -8 and -9 expression levels are important prognostic factors in CRC [33];[34]. Activation of caspase-8, which is induced by external signals, including chemotherapeutic agents, leads to apoptosis [35] mainly through the extrinsic pathway and may also activate the intrinsic apoptotic pathway through activation and cleavage of BID. Both pathways of apoptosis merge atone point in an apoptotic cellas both, caspase-9 and caspase-8 activate caspase-3 at the end [36]. The present results are therefore very encouraging as they show the effective activation of the three key apoptosis-inducing caspases.

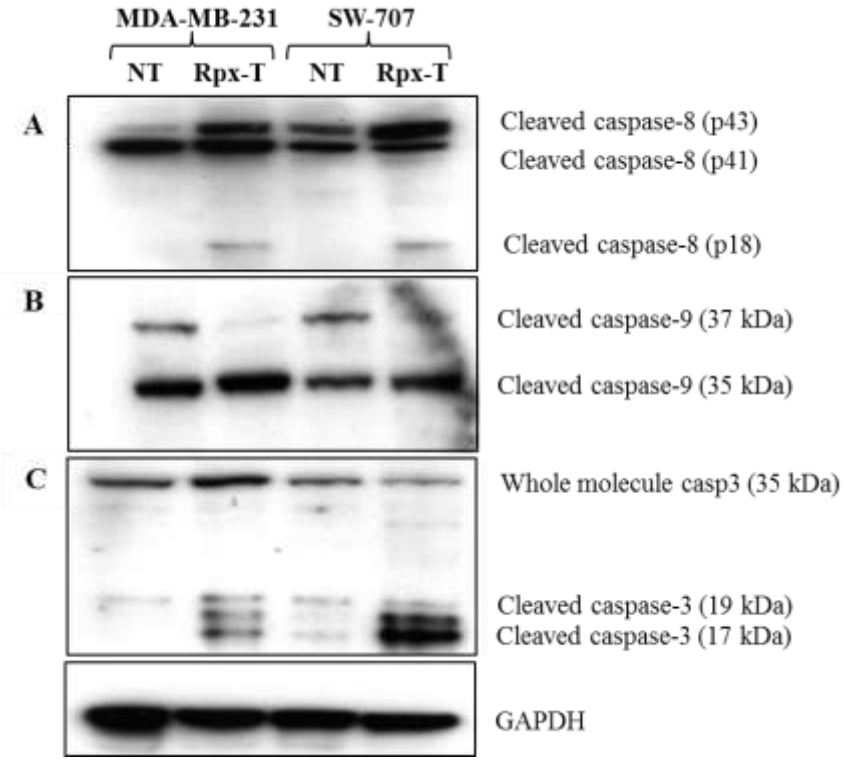

Figure 5: Protein expression of caspases (8, 9 and 3) in human MDA-MB-231 breast and SW-707 colorectal cancer cells. The cells were treated with corresponding IC_{50}concentrations for 48 h and caspases-3, -8 and -9 activation status were determined by western blot analysis. Caspase-8 was activated or cleaved after ligation of Fas receptor after which it activated caspase-3 leading to apoptosis by the extrinsic pathway. Caspase-9 was activated following release of cytochrome C by the mitochondria and cleaved caspase-9 activated caspase-3 leading to apoptosis by the intrinsic pathway.

Rpx activatesor inhibits components involved in cell apoptosisin breast and colorectal cancer cells

Following 48 h exposure of MDA-MB-231 and SW-707 cancer cells toRpx (IC_{50}), protein levels of key apoptosis players were either increased or decreased. In MDA-MB-231 cells, there was a slightly increased protein level of the adaptor FADD, which transmits death signals from Fas death receptor to caspase-8. As it is obvious from the decreased levels, the BH3 interacting-domain death agonist, or *BID,* was significantly converted to its active form or truncated BID (tBID). In fact, BID decreased by 81% in MDA-MB-231 cells and by 70% in SW707 cells in response to Rpx and concomitantly gave rise to tBID. This fragment may have interacted then with BAX and not BAK to cause release of cytochrome C from mitochondria. There was a significantly ($p \leq 0.05$) higher BAX protein expression in treated MDA-MB-231 cells as compared to untreated cells and a similar trend was observed for treated SW-707 cells. Furthermore, following 48 h treatment of MDA-MB-231 or SW-707 with Rpx, BAK protein expression did not differ significantly between non-treated and treated cells. Significantly higher cytochrome Cprotein levels were observed in both treated MDA-MB-231 and SW-707 cells when compared to the untreated cells ($p \leq 0.05$).Finally, Rpx treatment of MDA-MB-231 or SW-707 cells led to a significant ($p \leq 0.05$) inhibition of Bcl-2 protein expression in treated vs. untreated cells (Figure 6).

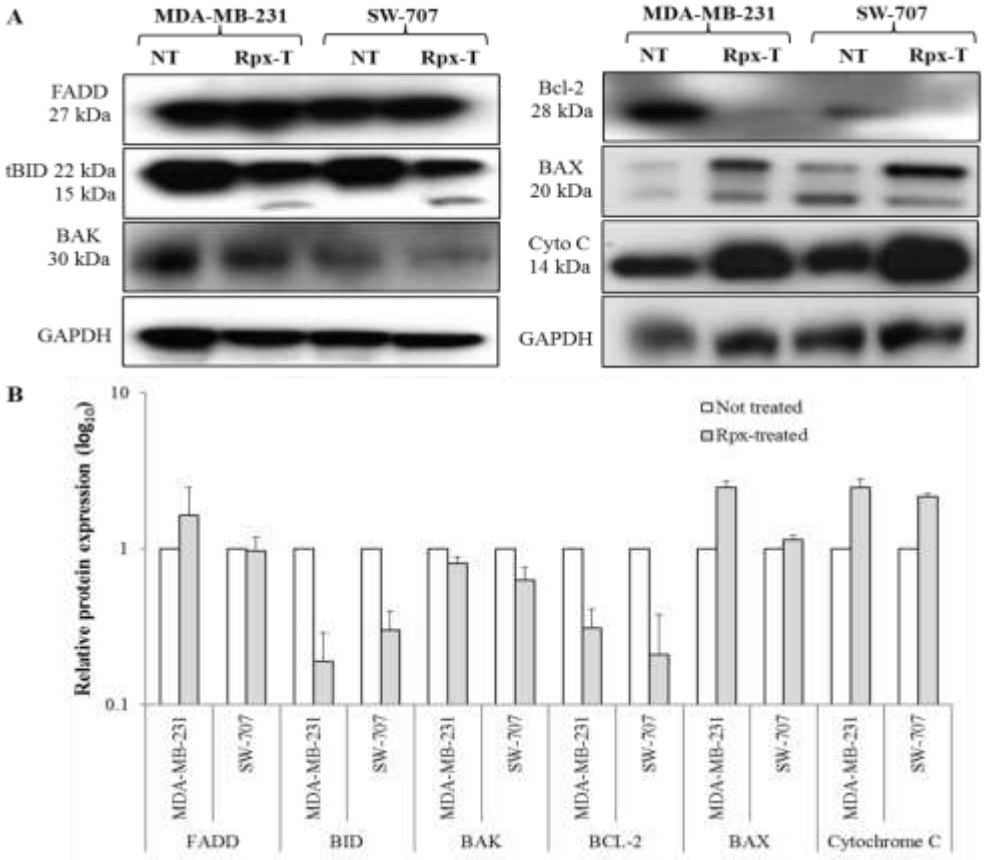

Figure 6: Protein expression of FADD, tBID, BAK, BCL-2, BAX and cytochrome C (CYTO C) as shown by Western blot in human MDA-MB-231 breast and SW-707 colorectal cancer cells following 48 h exposure to corresponding Rpx IC50 concentrations (A). Protein expression levels were calculated relative to GAPDH expression as a loading control (B).

Targeting and inducing apoptosis in tumor cells is one of the most important strategies for treatment of cancers and it has been established that promising anti-cancer agents are derived from plants and achieve their cancer killing activity by activating the apoptotic pathway [3]. The present study reports for the first time, the potential use of Rpx to kill cancer cells through activation of caspase-8,which in turn activates caspase-3 for terminal execution of treated cells through the extrinsic or death receptor pathway. In addition, the present findings demonstrate that BID was cleaved by the activated caspase-8, into tBID, the form of BID that associates with the mitochondrial outer membrane (MOM). This in turn caused pore formation or permeabilization of the membrane leading to release of cytochrome C. Importantly, this observation may guide in the choice of Rpx for the treatment of tumors because the two main pathways of apoptosis, the extrinsic and the intrinsic pathway, are being targeted.

BID has been shown to have a direct intermolecular contact with BAX in order to permeabilize the MOM at the initiation of the intrinsic pathway of apoptosis [37]. The observation of this study that BAX and not BAK was activated in Rpx-treated cancer cells, is a confirmation that tBID cooperated with BAX and not BAK to perforate the MOM leading to the observed release of cytochrome C. Of much interest is the significant increase in BAXand cytochrome Cprotein expression, signifying the superiority of Rpx as an anti-cancer agent important for triggering the cell apoptotic machinery. It has been widely established that once cytochrome Cis released into the cytosol, it interacts with ApaF-1 leading to activation of caspase-9[21]. In the present study, the observed activation of caspase-9 in the two Rpx-treated cell lines ended up in activating caspase-3, which executes the final apoptotic activities in target cells. The successful killing of Rpx-treated cancer cells must have been supportedby the ability of Rpx to significantly inhibit the expression of BCL2, both at gene and protein levels, while at the same time promoting the significant expression of BAX. Furthermore, it has been reported that upregulation of BCL2 expression and loss of BAX and/or BAK are the major means of anticancer drug failures and chemoresistance, while decreased expression promotes activity of anticancer agents [38];[39];[3] as confirmed in our present experiments.

Conclusion

The present study has demonstrated the potential use of Rpx as an anti-cancer agent that targetskey cellular components involved in regulation of cell proliferation and apoptosis. The study has clearly indicated that Rpx is able to inhibit cancer cell components involved in cell motility, cell invasion and metastasis such as NF-κB and ROCK1. The study has also revealed that Rpx can trigger both, the cytosolic and mitochondrial pathways of cell apoptosis by interacting with cell surface death receptors and by inhibiting the antiapoptotic- while at the same time activating the proapoptotic cellular components. Therefore, the present study adds new findings on the use of Rpx to activate both the extrinsic and intrinsic pathways of apoptosis using the human MDA-MB-231 and SW-707 as model cancer cell lines.

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