

Expression of genes and proteins of multidrug resistance in gastric cancer cells treated with resveratrol

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Received July 4, 2017; Accepted November 16, 2017

DOI: 10.3892/ol.2018.8022

Abstract. Multidrug resistance (MDR) is a notable problem in the use of chemotherapy. Therefore, studies aimed at identifying substances capable of overcoming resistance of cancer cells are required. Examples of these compounds are polyphenols, including resveratrol, that exert a range of various biological activities. The aim of the present study was to demonstrate the effect of 3,5,4'-trihydroxy-*trans*-stilbene (resveratrol) on the expression of ATP binding cassette subfamily B member 1, Annexin A1 (*ANXA1*) and thioredoxin (*TXN*) genes, and the proteins encoded by these genes, which are associated with MDR. The experiments were performed in human gastric cancer cell lines EPG85-257RDB (RDB) and EPG85-257RNOV (RNOV), which are resistant to daunorubicin and mitoxantrone, respectively, in addition to EPG85-257P (control), which is sensitive to cytostatic drugs. Cells were treated with 30 or 50 μ M resveratrol for 72 h and changes in the expression levels of the genes were analysed with the use of a reverse transcription-quantitative polymerase chain reaction. The cellular levels of P-glycoprotein (P-gp), *ANXA1* and *TXN* were evaluated using immunofluorescence and western blot analysis. Resveratrol in both concentrations has been shown to have a statistically significant influence on expression of the mentioned genes, compared with untreated cells. In RDB cells, resveratrol reduced the expression level

of all analyzed genes, compared with untreated cells. Similar results at the protein level were obtained for P-gp and *TXN*. In turn, in the RNOV cell line, resveratrol reduced *TXN* expression at mRNA and protein levels, compared with untreated cells. The results of the present study indicate that resveratrol may reduce the resistance of cancer cells by affecting the expression of a number of the genes and proteins associated with MDR.

Introduction

Resveratrol belongs to the stilbenoid group of polyphenols, possessing two phenol rings linked to each other by an ethylene bridge (1). In 1940, resveratrol was isolated from the root of *Veratrum grandiflorum* (1,2). A primary dietary source of resveratrol is red wine, as a very high resveratrol concentration is present in the skin of red grapes (50-100 μ g/g) (3). In natural conditions, resveratrol is synthesized by plants in response to external environmental factors, including ultraviolet radiation and heavy metals (3,4). Resveratrol is identified in two isomeric forms, *cis* and *trans*-resveratrol (Fig. 1) (5,6).

The *trans* form is dominant in terms of its prevalence and biological activity (7). The hydrophobic nature of resveratrol considerably contributes to its limited bioavailability (8). Owing to the varied biological activity of resveratrol, it has been the subject of numerous studies aimed at revealing its health-enhancing properties, and its use in prevention and treatment of many diseases (1,3,5,9). The results of *in vitro* and *in vivo* studies have proved that resveratrol exhibits anticancer activity at all three stages of the oncogenic process: initiation, promotion and progression (10).

Neoplastic diseases are presently one of the main causes of global mortality. One of the most commonly used cancer treatment methods is chemotherapy (11); however, its effectiveness is substantially reduced by multidrug resistance (MDR) (12-14). MDR is defined as the insensitivity to therapeutic substances that are not associated by structure or mechanism of action (15,16). The classical mechanism of MDR is associated with the overexpression of the ATP binding cassette subfamily B member 1 (*ABCB1*) gene encoding P-glycoprotein (P-gp), which contributes to the

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Abbreviations: P, human gastric carcinoma EPG85-257P cell line; RDB, human gastric carcinoma EPG85-257RDB cell line; RNOV, human gastric carcinoma EPG85-257RNOV cell line; R30, 30 μ M resveratrol; P-gp, P-glycoprotein; GUSB, β -glucuronidase; POLR2A, RNA polymerase IIa

Key words: multidrug resistance, resveratrol, gastric cancer

reduction of the effective drug concentration in the cell by transporting the drug out of the cell (14,15,17-20). In addition to the classical MDR mechanism associated with overexpression of P-gp, there are atypical mechanisms (21-23). Examples of these atypical mechanisms include the overexpression of Annexin A1 (ANXA1) and thioredoxin (TXN) proteins. The *ANXA1* gene encodes the ANXA1 protein, which reversibly binds Ca^{2+} ions and phospholipids of the plasma membrane (24). ANXA1 regulates cell growth, differentiation and apoptosis (25). Increased expression of *ANXA1* contributes to the formation of drug-filled vesicles, which are then exocytosed (26). The *TXN* gene encodes a protein involved in the process of the regulation of cellular redox state. In cancer cells, increased TXN expression results in an increase in proliferation and cell survival (27). Resveratrol is an example of a natural compound with the ability to overcome resistance to chemotherapy. *In vitro* studies on the sensitisation of cells to daunorubicin (DB) with the use of resveratrol, in cases of leukaemia, pancreatic and prostate cancer, it was revealed that resveratrol increased the apoptosis of those cells (28). The aim of the present study was to evaluate the effect of resveratrol on the expression level of *ABCBI*, *ANXA1* and *TXN* genes, which are associated with the MDR phenomenon in DB- and mitoxantrone (MTX)-resistant human gastric cancer cells.

Materials and methods

Cell lines and culture conditions. *In vitro* studies were performed in three human gastric cancer cell lines that were either sensitive or resistant to cytostatic drugs. EPG85-257P (P) cell line is sensitive to DB and MTX, whereas EPG85-257RDB (RDB) and EPG85-257RNOV (RNOV) cell lines are resistant to DB and MTX, respectively. Cell lines were obtained from the Institute of Pathology, Charité Campus Mitte, Humboldt University (Berlin, Germany). Cells were cultured in 75 cm² culture flasks (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Leibovitz's medium L-15 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), supplemented with 10% foetal bovine serum, 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/l transferrin, 1 g/l glucose, 1.1 g/l NaHCO_3 and 1% minimal essential vitamins (all from Sigma-Aldrich; Merck KGaA) was used to culture all cells. Adequate resveratrol (Sigma-Aldrich; Merck KGaA) was added to the medium to maintain resistance of the cells, 2.5 $\mu\text{g/ml}$ DB (Sigma-Aldrich; Merck KGaA) for RDB and 0.02 $\mu\text{g/ml}$ MTX (Sigma-Aldrich; Merck KGaA) for the RNOV cell line.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Changes in the expression of *ABCBI*, *ANXA1* and *TXN* genes in P, RDB and RNOV cell lines were evaluated following the use of resveratrol at concentrations of 30 μM (R30) and 50 μM (R50). Resveratrol concentrations were chosen based on cytotoxicity studies for this compound (29). After 72 h incubation at 37°C with resveratrol, the cells were trypsinized (37°C for 7 min; Sigma-Aldrich; Merck KGaA) and the resulting residues were collected. RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) was used for RNA isolation according to the manufacturer's protocol. To remove

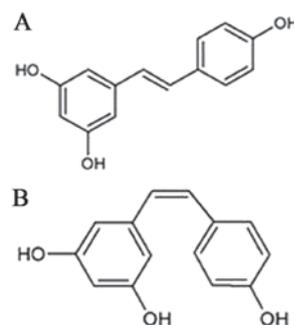


Figure 1. Structural formula of resveratrol isomeric forms. (A) *Trans*-resveratrol and (B) *cis*-resveratrol.

genomic DNA, samples were digested using the RNase-Free DNase Set (Qiagen GmbH), according to the manufacturer's protocol. Next, reverse transcriptase was performed using the High-Capacity cDNA Reverse Transcription kits (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Changes in gene expression were evaluated using RT-qPCR with the use of the 7900HT Fast Real Time PCR system, and SDS 2.3 and RQ Manager 1.2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Glucuronidase- β (*GUSB*) and RNA polymerase II subunit A (*POLR2A*) were used as reference genes. Relative expression of the genes in RDB and RNOV cells were assessed in comparison with the P cell line. The following sets of primers and TaqMan probes with FAMTM dye-labeled were used in the reactions: Hs00184500_m1 for *ABCBI*, Hs00167549_m1 for *ANXA1*, Hs01555212_g1 for *TXN*, Hs99999908_m1 for *GUSB* and Hs00172187_m1 for *POLR2A* (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling reactions were conducted in triplicate under the following conditions: polymerase activation at 50°C for 2 min, initial denaturation at 94°C for 10 min, and 40 cycles of denaturation at 94°C for 15 sec and annealing of the primers and probes and synthesis at 60°C for 1 min. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{Cq}}$ method (30). Three experimental replicates were performed.

Western blot analysis. Total cellular proteins were isolated from each of the tested cell lines at 4°C for 10 min, using 200 μl /3 mln cells with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% IGEPAL CA-630 and 0.5% sodium deoxycholate), 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail 10 $\mu\text{l/ml}$ RIPA. The samples were agitated for 30 min at 4°C, and centrifuged (4°C for 12 min at 12,000 x g) and the supernatant was collected. Protein concentration was determined using a bicinchoninic acid assay with the use of Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). A suitable quantity of 4X Glo Lysis buffer (31) was added to the remaining supernatant and was denaturated at 95°C for 10 min. Electrophoresis was performed by 10% SDS-PAGE at 140 V. Total cellular proteins were visualized with the Chemi Doc MP (Bio-Rad Laboratories, Inc., Hercules, CA, USA) apparatus. A total of 30 μg proteins (per lane) were transferred to polyvinylidene fluoride membrane (Immobilon-P; EMD Millipore, Billerica, MA, USA) in

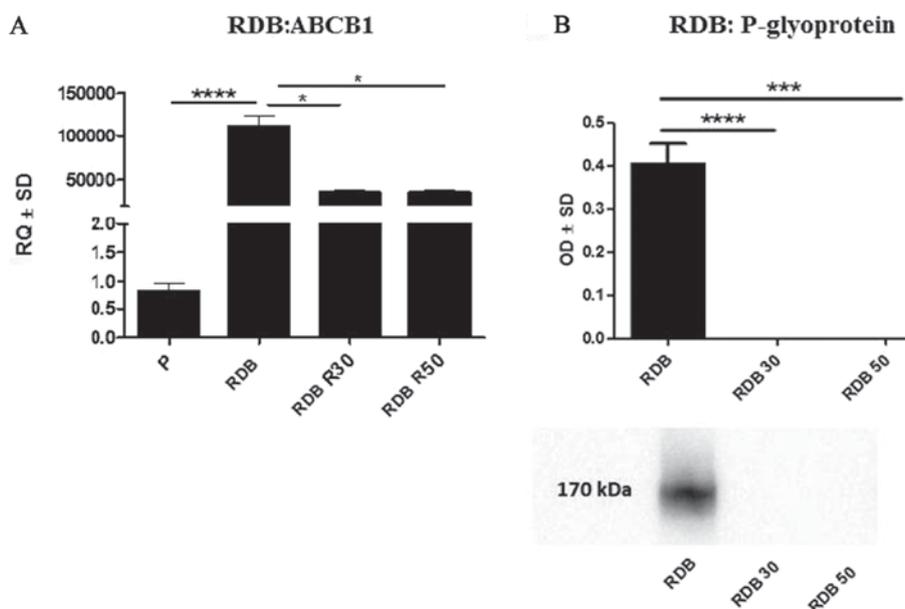


Figure 2. Effect of resveratrol on the levels of *ABCB1* and P-gp in a RDB cell line. Expression levels of (A) *ABCB1* gene and (B) P-gp protein in P and RDB cell lines. * $P < 0.05$ and **** $P < 0.0001$ with comparisons indicated by lines. *ABCB1*, ATP binding cassette subfamily B member 1; P-gp, P-glycoprotein; P, EPG85-257P cell line; RDB, EPG85-257RDB cell line; R30, 30 μM resveratrol; RQ, relative quantity; SD, standard deviation; OD, optical density.

transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol and 0.1% SDS, pH 9.2) by constant current at 250 mA for 1 h. Subsequent to washing the membranes in distilled water and 0.1% TBS-Tween-20 (TBST), they were blocked with 4% BSA (Sigma-Aldrich; Merck KGaA) in 0.1% TBST (room temperature for 1 h). Membranes were incubated with primary antibodies: Mouse anti-P-gp monoclonal antibody C219 (dilution 1:300; cat. no. ALX-801-002-C100; Enzo Life Sciences, Inc., Farmingdale, NY, USA), rabbit anti-ANXA1 polyclonal Ab H-65 (dilution 1:150; cat. no. sc-11387; Santa Cruz Biotechnology, Inc., Dallas, TX, US), rabbit anti-TXN monoclonal Ab (dilution 1:5,000; cat. no. LS-B7196; LifeSpan Biosciences, Inc., Seattle, WA, USA) at 4°C for 12 h. Secondary anti-mouse and anti-rabbit antibodies were used at a dilution of 1:3,000 at room temperature for 1 h. (CyTM 3 AffiniPure Donkey Anti-Rabbit IgG (H+L); cat. no. 711-025-152; and Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L); cat. no. 715-025-150; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The measurements were taken with the Chemi Doc MP System apparatus, using Image Lab 5.0 Software (both from Bio-Rad Laboratories, Inc.). The level of protein expression was determined as the ratio between signal intensity for a given protein and signal intensity for total cellular proteins.

Immunofluorescence (IF). The cells for the IF reaction were cultured at a density of 1×10^4 cells/well for RDB and 8×10^3 cells/well for RNOV in 8-wells Millicell[®] EZ SLIDES (Sigma-Aldrich; Merck KGaA). Culturing was performed under the same conditions as for RT-qPCR. The cells were fixed in 4% paraformaldehyde for 12 min at room temperature. For membrane permeabilisation, 0.2% Triton X-100 was used (for 10 min at room temperature). The cells were incubated for 1 h at room temperature with the following primary antibodies: Mouse anti-P-gp monoclonal antibody

C219 (dilution 1:100; cat. no. ALX-801-002-C100; Enzo Life Sciences, Inc.), rabbit anti-ANXA1 polyclonal antibody H-65 (dilution 1:100; cat. no. sc-11387; Santa Cruz Biotechnology, Inc.) and rabbit anti-TXN monoclonal antibody (dilution 1:100; cat. no. LS-B7196; LifeSpan Biosciences, Inc.). Proteins were detected with [tetramethylrhodamine (TRITC)-conjugated AffiniPure[®] donkey anti-rabbit IgG (H+L)] and TRITC-conjugated AffiniPure[®] donkey anti-mouse IgG (H+L) secondary antibodies (cat. nos. 711-025-152 and 715-025-150; Jackson's ImmunoResearch Laboratories, Inc.), at a dilution of 1:2,000 (1 h incubation at 4°C). Preparations were mounted on DAPI-containing media (Prolong Gold Antifade Reagent with DAPI; Thermo Fisher Scientific, Inc.). The analysis of protein expression was conducted using a fluorescent microscope (Olympus BX41; Olympus Corporation, Tokyo, Japan) at x40 magnification.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance for ranks (Kruskal-Wallis test) was performed using post-hoc Dunn's or Bonferroni tests. Data were presented as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of resveratrol on the level of expression of *ABCB1* gene and P-gp protein in an RDB cell line. The untreated DB-resistant RDB cell line significantly overexpressed the *ABCB1* gene when compared with the P cell line, which is DB-sensitive ($P < 0.0001$; Fig. 2A). The level of *ABCB1* gene expression was significantly reduced following the use of resveratrol at R30 concentration in comparison with expression in the untreated DB-resistant cell line ($P < 0.05$). A similar

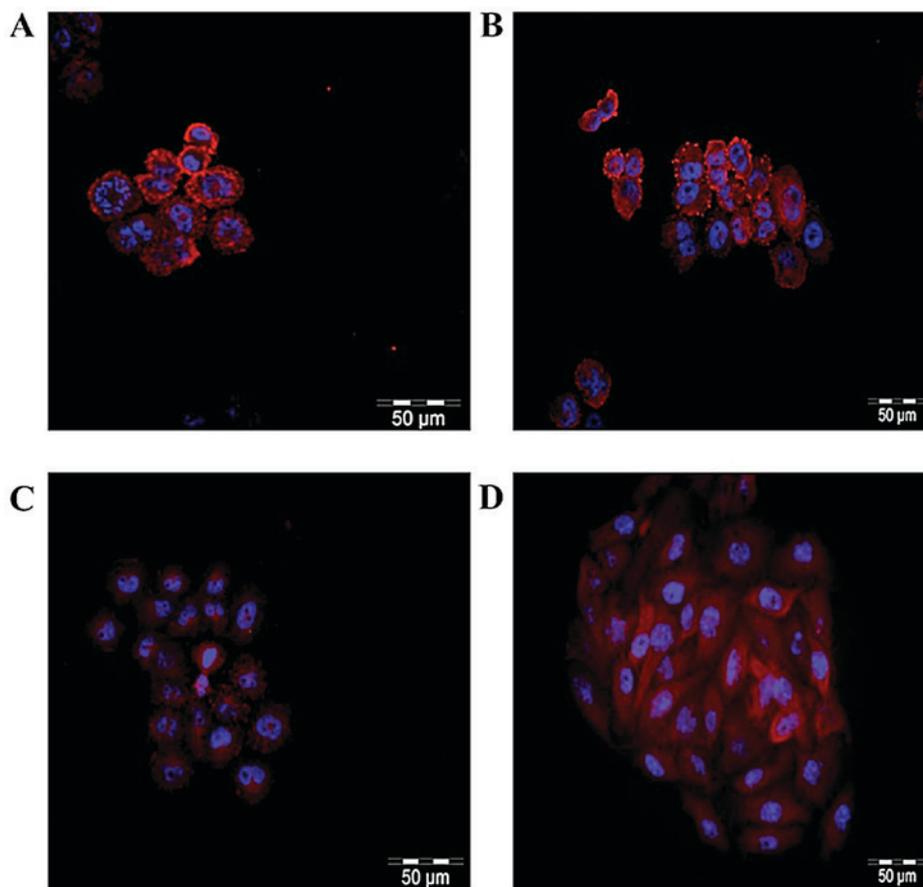


Figure 3. Immunofluorescence reactions confirming the presence of P-gp, ANXA1 and TXN in human gastric cancer cell lines. Presence of (A) membrane reaction, P-gp, primarily in the membrane; (B) ANXA1, primarily in the cytoplasm; (C) TXN, primarily in the cytoplasm, all in the EPG85-257RDB cell line; and (D) TXN, primarily in the cytoplasm in the EPG85-257RNOV cell line. P-gp, P-glycoprotein; SD, standard deviation; ANXA1, Annexin A1; TXN, thioredoxin.

association was revealed at the protein level with the use of western blot analysis. In the R30- and R50-treated groups, the protein level dropped below the quantitation level. Thus, there was a statistically significant difference between the two resveratrol-treated groups and the untreated group ($P < 0.0001$; Fig. 2B).

The presence of individual proteins in cell lines was confirmed using IF. In the RDB cell line: P-gp (Fig. 3A); Annexin A1 (Fig. 3B); and thioredoxin (Fig. 3C), and in the RNOV cell line, thioredoxin (Fig. 3D).

Effect of resveratrol on the change of the expression of ANXA1 and TXN genes and proteins in the RDB cell line. It was revealed that in the RDB cell line, aside from the classical resistance mechanism (*ABCB1* gene overexpression), other atypical mechanisms are present that are associated with an increased expression of *ANXA1* and *TXN* genes. Expression of the *ANXA1* gene in RDB cells is significantly higher than that in P cells ($P < 0.0001$; Fig. 4A). R30 treatment resulted in the significant reduction of gene expression in the RDB cell line compared with the P cell line ($P < 0.001$; Fig. 4A). R50 treatment resulted in the additional decrease in the expression of this gene, significantly when compared with control RDB cells ($P < 0.001$) and non-significantly compared with R30-treated RDB cells (Fig. 4A). On the protein level, an increase in the expression of ANXA1 with increasing

resveratrol concentration was revealed; however, this difference was not statistically significant (Fig. 4B). However, with respect to the *TXN* gene, significant overexpression was observed in the RDB cell line in comparison with the P cell line ($P < 0.001$; Fig. 5A). Incubation with R30 resulted in the significant reduction of gene expression when compared with the control RDB cell line ($P < 0.001$; Fig. 5A). Analysis of protein levels confirmed the results of RT-qPCR. Treating RDB cells with R30 resulted in a reduced level of protein expression when compared with RDB cells not treated with the polyphenol, with R50 resulting in the further reduction of TXN expression compared with non-treated cells and R30-treated cells (Fig. 5B).

Effect of resveratrol on the expression level of TXN mRNA and protein in the RNOV cell line. The presence of atypical MDR mechanisms associated with the overexpression of *TXN* was revealed in the RNOV cell line. Expression of this gene in the RNOV cell line was significantly higher in comparison with the P cell line ($P < 0.001$; Fig. 6A). R30 significantly reduced the level of gene expression in comparison with the untreated RNOV cell line ($P < 0.0001$; Fig. 6A). The same results were obtained at the protein level: The difference in expression levels between TXN in untreated RNOV cells and R50-treated RNOV cells was statistically significant ($P < 0.05$; Fig. 6B).

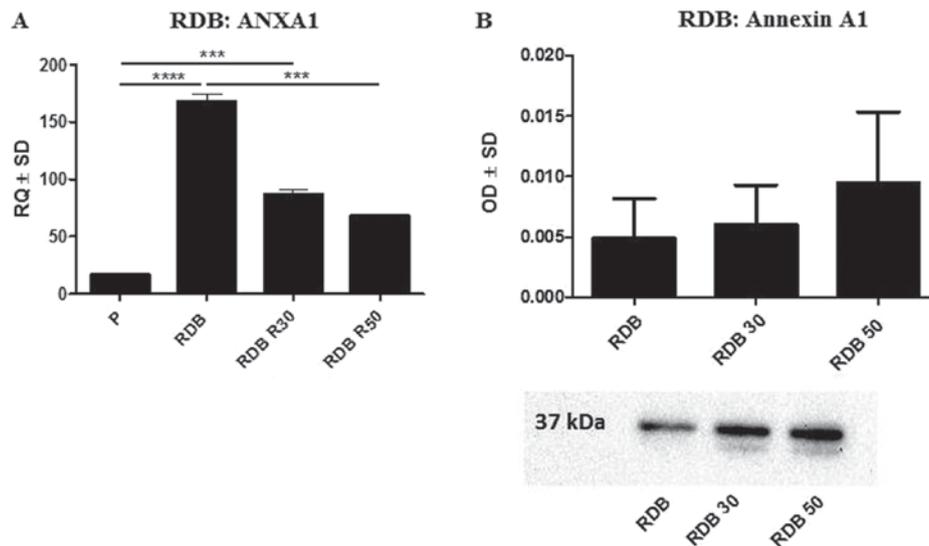


Figure 4. Effect of resveratrol on the expression levels of *ANXA1* and ANXA1. Expression levels of (A) *ANXA1* and (B) ANXA1 protein in P and RDB cell lines. *** $P < 0.001$ and **** $P < 0.0001$, with comparisons shown by lines. R30, 30 μM resveratrol concentration; R50, 50 μM resveratrol; P, EPG85-257P; RDB, EPG85-257RDB; *ANXA1*, Annexin A1; RQ, relative quantity; OD, optical density; SD, standard deviation.

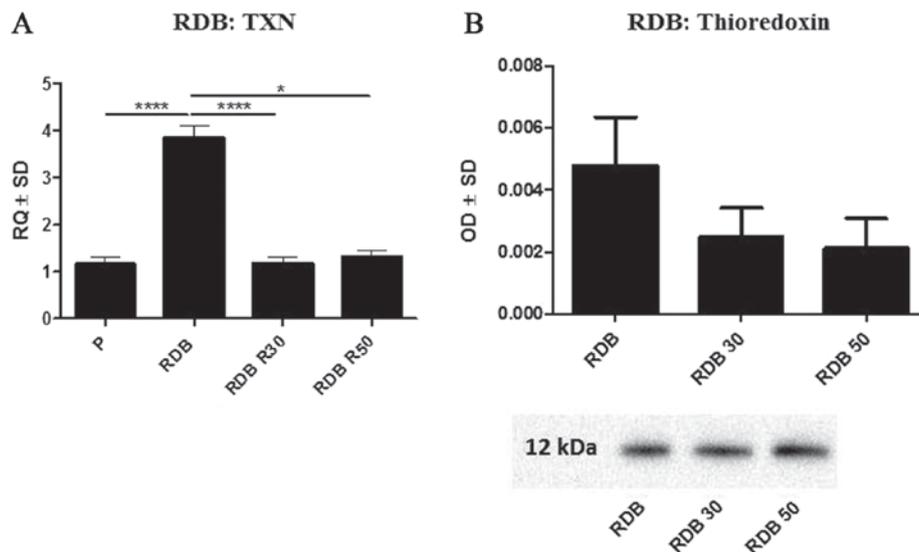


Figure 5. Effect of resveratrol on the expression levels of *TXN* and TXN in RDB cells. Expression levels of (A) *TXN* gene and (B) TXN protein in P and RDB cell lines. * $P < 0.05$ and **** $P < 0.0001$. R30, 30 μM resveratrol concentration; R50, 50 μM resveratrol; P, EPG85-257P; RDB, EPG85-257RDB; *TXN*, thioredoxin; RQ, relative quantity; OD, optical density; SD, standard deviation.

Discussion

MDR is a notable problem for chemotherapy and results in a lower chemotherapeutic efficacy (32). Drug-resistance in cancer cells develops as a result of numerous different mechanisms, of which understanding has improved owing to the use of novel research techniques. These mechanisms include the lowering of the intracellular drug concentration, activation of detoxification enzymes, changes in drug metabolism inside of the cell and the inhibition of cancer cell apoptosis (33,34). The most well-known and frequently described MDR mechanism is the increased expression of transport proteins at the membrane of cancer cells (35). Therefore, studies on substances that enable cancer cell sensitisation to cytostatics

are necessary. Such compounds include polyphenols such as resveratrol, curcumin or quercetin (19,36). The effects of polyphenol compounds were studied in human leukaemia cell lines sensitive (K562) and resistant (K562/A02) to DB. In this study, it was revealed that curcumin contributes to the reduction of cell viability and to DNA damage. The effect of curcumin on cancer cell apoptosis was also reported (37). Resveratrol and its ability to overcome MDR were studied in pancreatic cancer cell lines EPP85-181 that were sensitive or resistant to cytostatics, in which the low level of type II topoisomerase expression was one of the causes of resistance. Resveratrol increased the expression of type II topoisomerase and therefore enhanced cells sensitivity to antitumor agents (36).

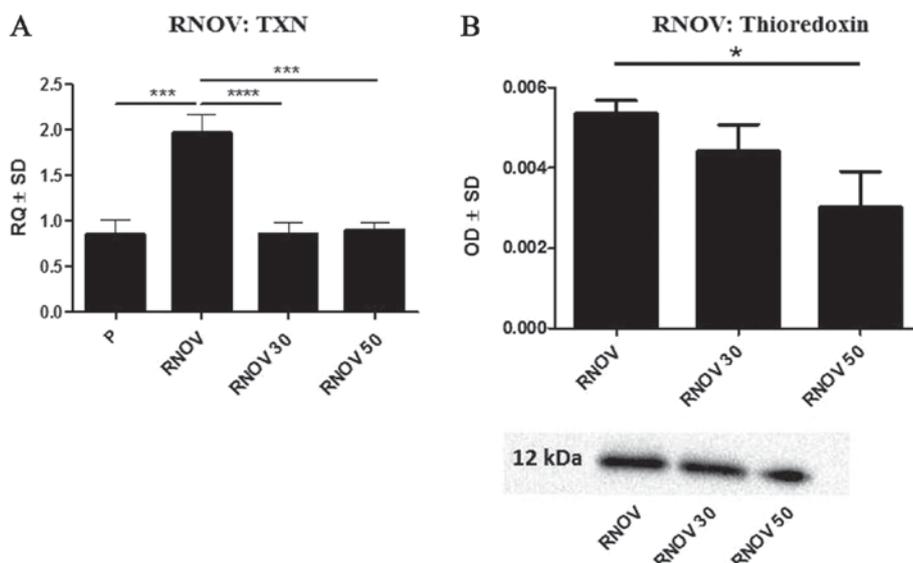


Figure 6. Effect of resveratrol on the expression levels of *TXN* and TXN in RNOV cell lines. Expression levels of (A) *TXN* gene and (B) TXN protein in P and RNOV cell lines. * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$. R30, 30 μM resveratrol; R50, 50 μM resveratrol concentration; P, EPG85-257P; RNOV, EPG85-257RNOV; *TXN*, thioredoxin; RQ, relative quantity; OD, optical density; SD, standard deviation.

The present study attempted to demonstrate the effect of resveratrol on the expression of MDR-associated *ABCB1*, *ANXA1* and *TXN*, and the proteins encoded by these genes.

The ability of P-gp to actively remove drugs used for chemotherapy, including DB, results in a low intracellular concentration of these drugs and therefore treatment failure (14,38). The effect of resveratrol was studied in human colorectal cancer HCT116 cell lines that were sensitive or resistant to oxaliplatin (39). In this study, it was revealed that resveratrol treatment results in a reduction in *ABCB1* expression in cisplatin-resistant cells in comparison with the expression in cisplatin-sensitive cells, by inhibiting nuclear factor- κB (NF- κB) and AMP-activated protein kinase-dependent activation of cAMP response element binding protein. The effect of resveratrol on P-gp expression in the human epidermal carcinoma KBv200 cell line was additionally investigated, and it was demonstrated that resveratrol efficiently reduced the expression of this protein (40). The present study revealed the increased expression of the *ABCB1* gene and P-gp in the DB-resistant RDB cell line in comparison with a DB-sensitive cell line. Resveratrol significantly reduced P-gp expression at the mRNA and protein level in a concentration-dependent fashion. In the MTX-resistant RNOV cell line, no increased expression of P-gp or *ABCB1* was observed, indicating that an atypical resistance mechanism was being observed. A previous study demonstrated that, in cancer cells resistant to cytostatics the mechanism of action of curcumin is to inhibit P-gp in a competitive or allosteric manner (41).

Until now, to the best of our knowledge, the effect of resveratrol on *ANXA1* gene expression in cancer cells remained unstudied. A previous study examined the influence of resveratrol on *ANXA1* expression in an acute promyelocytic leukaemia HL-60 cell line, in which it was revealed that the protein level increased depending on the time of incubation with the polyphenol (42). *ANXA1* inhibits proliferation and activates apoptosis by affecting the extracellular signal-regulated kinase signaling cascade, binding actin

filaments and stimulating cyclin-dependent kinase 2 (43). The main pro-apoptotic mechanism of *ANXA1* is most likely via caspase-3 activation (44,45). In addition to the classical MDR mechanism, atypical mechanisms are associated with increased *ANXA1* expression in the RDB cell line (46). In the present study, an increase in the expression of *ANXA1* in RDB cells compared with that in a DB-sensitive cell line was revealed. Treatment with resveratrol reduced the expression level of *ANXA1*. However, in MTX-resistant cells, high expression of *ANXA1* was not observed. At the protein level, treating cells with resveratrol resulted in an increase in *ANXA1* expression in DB-resistant cells compared with cells that are DB-sensitive. These results are in line with those obtained by Li *et al* (42).

The results of studies performed using various types of cancer revealed that *TXN* gene expression is higher in cancer cells than in non-cancer cells (47). Using two-dimensional gel electrophoresis, *TXN* expression was observed in an MTX-resistant RNOV cell line (46). The high level of expression of this gene may be associated with increased proliferation, resulting in cancer cell resistance to chemotherapy (48). These results are consistent with those of the present study, which found that *TXN* is overexpressed in RDB and RNOV cell lines when compared with their expression in drug-sensitive cell lines. Upon incubation of the cells with resveratrol, the level of expression of *TXN*, and that of the protein it encodes, was reduced.

The lowered expression of TXN may induce apoptosis; the key element is the TXN-TXN reductase system, which maintains a proper redox state (49). Deficiency of this protein affects compounds serving notable functions in the cancer transformation process. Amongst the most crucial compounds is the NF- κB transcription factor, which is responsible for the transcription of B-cell lymphoma 2 family genes; products of this gene family additionally inhibit apoptosis (49). The decrease in TXN expression results in the inhibition of activation and pro-apoptotic action further apoptosis signal-regulating kinase-1 binding (50).

In summary, resveratrol is a compound that is able to overcome MDR in human gastric cancer cells. By affecting the expression of genes and proteins that are crucial for MDR, it may contribute to an increase in the efficiency of chemotherapy.

Acknowledgements

The authors would like to thank Professor H. Lage and Professor M. Dietel (Humboldt University, Berlin, Germany) for providing the EPG85-257 cell lines used in the present study.

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