Classical and atypical resistance of cancer cells as a target for resveratrol

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Abstract. The phenomenon of cancer cell resistance to chemotherapeutics is the main cause of insensitivity to anticancer therapy. Thus, the current challenge remains searching for substances sensitising the activity of cytostatic drugs. In this respect, resveratrol is a very promising therapeutic agent. It has pleiotropic effect on cancer cells, which can play a key role in numerous resistance mechanisms, both classical and atypical. The purpose of the present study was to assess the effect of resveratrol on the inhibition of human pancreatic cancer cell proliferation and on the level of cytostatic resistance-associated proteins. The study was performed on human pancreatic cancer cell lines EPP85-181P (control), EPP85-181RDB (daunorubicin resistance) and EPP85-181PRNOV (mitoxantrone resistance). The effect of resveratrol on the viability and proliferation of the studied cell lines was evaluated by SRB assay, whereas cell cycle arrest and cytostatic accumulation by FACS. Western blot analysis was used to determine the level of P-glycoprotein, topoisomerase II α and β and immunofluorescence technique to visualise the proteins in the cells. Resveratrol inhibited proliferation of all studied cell lines. Phase-specific cell cycle arrest depended on the type of cancer cells. Resveratrol decreased the level and activity of P-gp in EPP85-181RDB cells. In EPP85-181PRNOV cells, expression of both TopoII isoforms increased in a statistically significant manner. The results of *in vitro* studies support the possibility of potential use of resveratrol in breaking cancer cell resistance to chemotherapeutic drugs.

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Abbreviations: P, parental cells of human gastric carcinoma line EPP85-181P; RDB, resistant cells of human gastric carcinoma line EPP85-181RDB; RNOV, resistant cells of human gastric carcinoma line EPP85-181RNOV; R30, resveratrol concentration of 30 μM; R50, resveratrol concentration of 50 μM; SRB, sulforhodamine B; P-gp, P-glycoprotein; TopoII, topoisomerase II

Key words: multidrug resistance, resveratrol, P-glycoprotein, topoisomerase II, pancreatic cancer

Introduction

Resveratrol (3,5,4'-trihydroxystilbene) belongs to the plant polyphenols from the group of stilbenes. Natural sources of this substance are edible plants such as grapes, peanuts or mulberries. Particularly high amounts of resveratrol can be found in grape skins, therefore, it is considered to be the key compound responsible for so-called 'French paradox'. *In vitro*, *in vivo* and epidemiological studies show that there is a link between red wine consumption in France and protection against cardiovascular diseases, predominantly in individuals on a diet that is high in saturated fats (1-3). Because of its positive effect on human health, for many years resveratrol has been of interest to many researchers all over the world. Among its properties that deserve the highest attention are anti-inflammatory, anti-viral, anti-bacterial, anti-aging and anti-carcinogenesis activities, as well as reducing level of LDL cholesterol. Inhibitory effect of resveratrol on all stages of carcinogenesis was described. This was proved by numerous studies, both pre-clinical (*in vitro* and *in vivo*) and clinical (4-9). Resveratrol's mechanisms of action on cancer cells are multidirectional. These can be direct interactions by influencing certain genes, transcription factors and enzymes, as well as indirect interactions affecting important biochemical pathways in the cells. Dose, exposure time and cell type-dependent anticancer effect of resveratrol was proved, among others, on cell cycle, apoptosis, autophagy, adhesion and intercellular signalling, detoxication and DNA repair processes (5,10-12). Additionally, there have been reports that resveratrol can sensitise cancer cells to cytostatics in various types of chemotherapy resistance. Very low toxicity and protective effect on normal cells are additional values of this polyphenol (13-16).

Multidrug resistance (MDR) is a serious problem in chemotherapy. MDR phenomenon arises from insensitivity of cancer cells to various types of drugs that often differ from each other in structure and mode of action. Mechanisms of MDR can be of extracellular nature, e.g. poor tumour vasculature or pH

of extracellular matrix, or intracellular. There are very many reasons why cancer cells do not respond to chemotherapy. Usually, intracellular mechanisms can be divided into classical and atypical. The classical ones concern P-glycoprotein (P-gp), the best known and the most often described ABC transporter associated with MDR, which actively pump-out drug particles from the cell. P-gp is multi-domain membrane protein having molecular mass of 170 kDa and encoded by *ABCB1* gene. Overexpression of P-gp is the main factor determining resistance of cancer cells to many cytotoxic substances having different structures and properties (17,18). Other causes of resistance are considered 'atypical', however, it does not mean that they occur only occasionally. Most of the time, several mechanisms of MDR are active simultaneously. Examples are alterations in the level of cellular pathways involved in various types of programmed cell death (e.g. overexpression of anti-apoptotic proteins) or DNA repair (e.g. proteins involved in mismatch repair, base or nucleotide excision repair) and other molecular mechanisms (e.g. level of topoisomerase II expression) (19-21). Topoisomerase II (TopoII) is an enzyme playing an important role in replication, transcription, recombination, as well as chromosome structure and segregation. The main role of TopoII is cleavage of both DNA strands and their movement, followed by ligation of phosphodiester bonds. Therefore, it is molecular target point for cytostatics. There are two isoforms of human TopoII, α and β (22). Those enzymes are overexpressed in numerous types of tumours. Unfortunately, lower level of TopoII may cause resistance, particularly to anthracycline group of drugs. It is also known that homozygous deletion of gene encoding the α isoform of *TOPO2A* may significantly affect the effectiveness of anthracycline therapy e.g. in breast cancer (23,24).

In case of pancreatic cancer prognosis are particularly poor, mainly because of late detection, rapid progress of the disease and the resistance of cancer cells to chemotherapy and radiotherapy (25,26). Morbidity to this type of cancer is high, but survival is very low and in Europe it is estimated at 4.6 months from diagnosis. Recently it was proved that polyphenols, including resveratrol, inhibit pancreatic cancer development at all stages of tumour growth, during initiation, progression, metastasis and invasion. Moreover, it may affect pancreatic cancer stem cells by inhibition of pluripotency-maintaining factors and epithelial-mesenchymal transition. Additionally it can sensitise cancer cells to cytostatics (27,28).

The purpose of our studies was to evaluate *in vitro* the effect of resveratrol on cells of human pancreatic cancer, with particular attention being paid to the expression of proteins responsible for resistance to cytostatics. Cellular models used in the research are characterised by different mechanisms of MDR, which gives the possibility to verify potential multidirectional activity of resveratrol.

Materials and methods

Cell culture and drugs. Human pancreatic cancer cell line EPP85-181P (P), parental cells and its drug-resistant derivatives were the *in vitro* model system for the study. The cells were grown in L-15 medium (Lonza, Gdańsk, Poland) supplemented with 10% fetal bovine serum (FBS; Lonza) and 10% FBS, 1 mM L-glutamine, 80 IE/l insulin, 6.25 mg/l fetuin, 2.5 mg/l transferrin, 1.1 g/l $NaHCO_3$, 1 g/l glucose, 1% minimal essential vitamins (Sigma-Aldrich, Darmstadt, Germany). The resistant cell line EPP85-181RDB (RDB) was grown in the presence of 2.5 μg/ml daunorubicin (DB) and EPP85-181RNOV (RNOV), 0.02 μg/ml mitoxantrone (MTX). Cell culture was performed as previously described (29). In RDB cell line the basic mechanism of resistance is the overexpression of P-gp, whereas in RNOV cell line resistance is mainly caused by reduced level of TopoII. The following agents were used: doxorubicin, mitoxantrone and resveratrol (Sigma-Aldrich). The therapeutic dose of cytostatics used in the experiments (the concentration of the cytostatic drug in patient's blood 2 h after administration): DB 0.25 μg/ml and MTX 0.02 μg/ml.

Cytotoxicity assay. In each assay, $1.5x10^4$ cells of P, RDB and RNOV lines were seeded in 96-well plates (TTP Techno Plastic Products, Trasadingen, Switzerland) 24 h prior to the experiment. Incubation with resveratrol (1-500 μM) was performed for 72 h. Cells were trypsinised and resveratrol cytotoxicity was examined using sulforhodamine B (SRB) assay (30). Absorbance was quantified at 562 nm. In order to determine IC_{50}-value, the absorbance difference of resveratrol untreated control cells was set to 100%. Linear regressions were plotted and IC_{50}-values were calculated for each cell line.

Cell cycle analyses by flow cytometry (FACS). Cells of each cell line ($2x10^5$ cells/well) were cultured in 6-well tissue culture plates (EuroClone, Milan, Italy) in specific Leibovitz L15 medium (Sigma-Aldrich) for 24 h at 37˚C, 5%, CO_2. After 24 h, cells were treated with resveratrol at a concentration of 30 μM (R30) and 50 μM (R50) for 72 h. Control and resveratrol-treated cells were trypsinised with 0.25% Trypsin-EDTA (Sigma-Aldrich), centrifuged (Thermo Fisher Scientific GmbH, Dreieich, Germany) at 1,000 rpm for 5 min at room temperature, and washed twice in PBS. Then, the cells were resuspended in ice-cold PBS and fixed overnight in 70% ethanol at 4˚C. Cells were pelleted by centrifugation (1,000 rpm, 5 min, 4˚C), washed twice in PBS and resuspended in a small amount of phosphate-buffered saline at room temperature. Samples of each cell line were mixed with propidium iodide (PI) and RNase staining solution (Life Technologies, Carlsbad, CA, USA) and incubated at 37˚C in the dark for 30 min. PI fluorescence was measured in the FL-2 channel of the BD FACSCanto II flow cytometer (BD Biosciences, Temse, Belgium). Data from minimum 20,000 events per sample were collected and calculated with ModFit LT™ software, version 4.0.5 (Verity Software House, Inc., Topsham, ME, USA). The experiment was performed in 3 independent replications.

Drug accumulation assay. Measurement of cellular daunorubicin accumulation was performed by flow cytometry using FACSCanto II supported by BD FACSDiva (version 6.1.3) analysis software (BD Biosciences, San Jose, CA, USA).

The cells of the P and RDB cell lines were transferred to 6-well plates (EuroClone), $4x10^5$ cells/well. After 24-h incubation at 37˚C and under 5% CO_2 atmosphere, the medium was removed from all wells. Next, the culture medium was applied to the non-treated control cells (C) and resveratrol

at concentrations of R30 and R50 μM was added to the remaining wells. Cells were incubated for 72 h at 37˚C. Then daunorubicin 2.5 μg/ml was added and after 2 h cells were harvested by trypsinisation, centrifuged (1,000 rpm, 5 min, 24˚C) and washed twice in PBS. Next, the cells were resuspended in 200 μl ice-cold BSA buffer (1% bovine serum albumin in 1X PBS) and stored on ice until the intracellular fluorescence of daunorubicin was measured in the FL-2 channel. A minimum of 100,000 cells were collected for each sample and the experiments were performed in six independent replications. In order to analyse the cell cycle, the total number of cells was counted, obtaining the result of 20,000 events. Positive events, from which the fluorescence signal was measured, represent the population of cells that accumulated daunorubicin in their interior. The experiment was performed in 6 independent replications.

Immunofluorescence analyses. Each of the cell lines were cultured in densities of $6x10^3$ cells/well on 8-well Merck Millicell EZ slides (Merck Millipore, Darmstadt, Germany). After 24 h, cells were treated with resveratrol (Sigma-Aldrich) at concentrations of R30 and R50 for 72 h. Afterwards, the slides were washed twice in PBS, fixed in 4% formaldehyde in PBS (12 min, room temperature) and then incubated with 0.2% Triton X-100 in PBS (10 min, room temperature). The fixed cells were incubated overnight at 4˚C with specific antibodies. Detection of protein expression was performed by using mouse monoclonal mAb against P-gp, clone C219 (1:2,000; Alexis Biochemicals, Lausen, Switzerland), rabbit monoclonal TopoIIα, clone D10G9 (Cell Signaling, Danvers, MA, USA) and rabbit monoclonal TopoIIβ, clone EPR5377 (Novus Biologicals LLC, Littleton, CO, USA). The primary antibodies were detected after 1 h of incubation with a donkey anti-mouse or donkey anti-rabbit secondary antibody, respectively, conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) at a dilution 1:2,000 in antibody diluent (Dako, Glostrup, Denmark). Finally, the slides were washed 3 times in PBS and mounted on ProLong Gold Mounting Medium (Invitrogen) with DNA intercalating dye 4,6-diamidino-2-phenylindole (DAPI) added to visualize the cell nucleus. The analysis of the results was conducted under fluorescent microscope, (Olympus BX51; Olympus, Tokyo, Japan) magnification x200. The data were collected using Cell-F software (Olympus).

Western blot analyses. Cells were transferred to cell culture flasks (25 cm^2), incubated for 72 h with resveratrol and/or combinations of resveratrol and cytostatics at 37˚C. Then, the cells were harvested by trypsinisation and centrifuged (1,000 rpm, 5 min, 24˚C), resuspended in PBS and washed twice.

Preparation of nuclear protein fraction for the TopoIIβ evaluation: resveratrol-treated cells were suspended in ice-chilled sterile isotonic buffer (50 mM Tris-HCl pH 7.6; 5 mM $MgCl_2$; 50 mM NaCl; 250 mM sucrose) and disrupted using needle and syringe. After centrifugation (10 min, 12,000 g, 4˚C) pellet of cells was lysed in the ice-cold lysis buffer (50 mM Tris-HCl pH 7.6; 250 mM NaCl; 1% Igepal NP-40; 0.5 mM PMSF and protease inhibitor cocktail) and centrifuged as described above. The supernatant was collected and protein concentration was determined by BCA method (according to the manufacturer's protocol; Thermo Fisher Scientific, Waltham, MA, USA).

Preparation of total cell lysate for the P-gp and TopoIIα evaluation: whole cell extracts were prepared by lysing resveratrol-treated cells in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5; 250 mM NaCl; 0,1% Igepal NP-40; 0.5 mM PMSF and protease inhibitor cocktail). After centrifugation (as described above), the supernatant was collected and protein concentration was determined by BCA method.

The cell lysates for the evaluation of TopoIIα and TopoIIβ levels were denaturised (10 min, 95˚C), while those for P-gp were incubated for 20 min in room temperature and then for 10 min in 4˚C. Equal amounts of proteins (30 μg) were subjected to SDS-PAGE by using 10% Mini-PROTEAN TGX Ready Gels (Bio-Rad Laboratories, Hempstead, UK) (31) and transferred to a PVDF Immobilon-P membranes (Millipore, Billerica, MA, USA) (32). After blocking (P-gp: 1 h, room temperature, 1% BSA in TBS, 0.1% Tween-20; TopoIIα: 1 h, room temperature, 4% BSA in TBS, 0.1% Tween-20; TopoIIβ: overnight, 4˚C, 4% BSA in TBS, 0.1% Tween-20), membranes were incubated overnight at 4˚C with specific antibodies: the mouse anti-P-gp mAb (C-219, 1:300; Alexis Biochemicals, San Diego, CA, USA), the rabbit anti-TopoIIα mAb (D10G9, 1:1,000; Cell Signaling Technology) and the rabbit anti-TopoIIβ mAb (EPR5377, 1:10,000; Novus Biologicals). Horseradish peroxidase-labelled secondary antibodies were incubated with the Immun-Star HRP Substrate (Bio-Rad Laboratories), visualized with ChemiDoc XRS Molecular Imager (Bio-Rad Laboratories) and normalized according to the β-tubulin (the mouse mAb, ab6046; Abcam, Cambridge, MA, USA). OD measurements of the protein bands were performed with the Image Lab software (Bio-Rad Laboratories).

Statistical analysis. Statistical analysis was performed using the Prism 5.0 software (Graphpad Software, Inc., La Jolla, CA, USA). When two groups of data were compared, the unpaired t-test was used. In cases of 3 or more groups, the one-way ANOVA with post-hoc analysis using the Dunnett's, Dunn's or Bonferroni multiple comparison tests were applied to compare the data among tested cell lines and experimental conditions. In all the analyses, the differences were regarded as significant when $P<0.05$.

Results

Cytotoxicity and cell cycle. Based on resveratrol cytotoxicity tests, IC_{50} was estimated for each of the studied cell lines to be: P, 158 μM, RDB, 343 μM and NOV, 269 μM. On the basis of statistical analysis it was determined that significant ($P<0.001$) proliferation inhibition occurred for 30 μM resveratrol (in comparison to control), and then for 50 μM (in comparison to 30 μM). Those two concentrations, 30 μM (R30) and 50 μM (R50), were selected for further studies.

Cell cycle analysis (Fig. 1) showed that in case of cell line sensitised upon administration of R50, the number of cells in S phase increased in comparison to control ($P<0.001$) with simultaneous decrease in other phases, particularly in G1. In DB-resistant cell line (RDB), increase in the number of cells arrested in G1 phase ($P<0.01$) upon incubation with R30 was observed. In case of MTX-resistant line (RNOV), significant increase in the number of cells in S phase ($P<0.001$) and G2/M

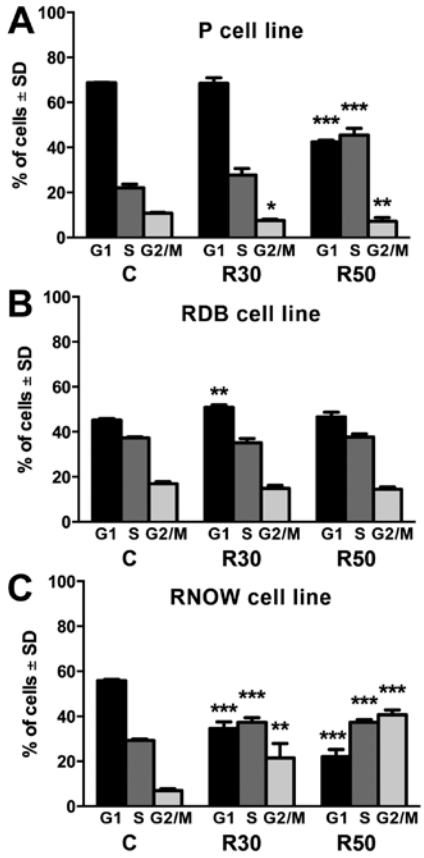

Figure 1. Cell cycle analysis. C, control; R30, cells treated with resveratrol at a concentration of 30 μM; R50, cells treated with resveratrol at a concentration of 50 μM. Analysis using Dunnett's test. (A) Cell line P: G1 - C vs. G1 - R50 (***P<0.001); S - C vs. S - R50 (***P<0.001); G2/M - C vs. G2/M - R30 (*P<0.05); G2/M - C vs. G2/M - R50 (**P<0.01). (B) Cell line RDB: G1 - C vs. G1-R30 (**P<0.01). (C) Cell line RNOV: C vs. G1 - R30 (***P<0.001), C vs. G1 - R50 (***P<0.001), S - C vs. S - R30 (***P<0.001), S - C vs. S - R50 (***P<0.001), G2/M - C vs. G2/M - R30 (**P<0.01), G2/M - C vs. G2/M -R50 (***P<0.001).

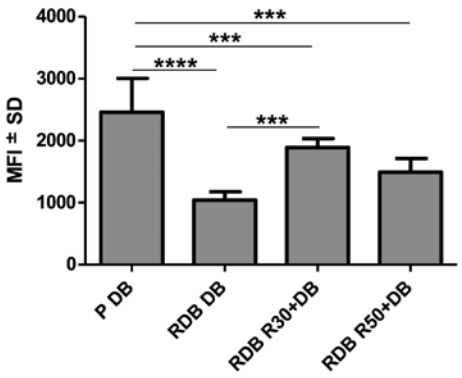

Figure 2. Daunorubicin accumulation measured by flow cytometry in P and RDB cell lines treated with daunorubicin (DB) in combination with resveratrol (R) at concentrations of 30 μM (R30) and 50 μM (R50). Data are presented as mean florescence intensity ± standard deviation (SD). ***P<0.001, ****P<0.0001, Bonferroni's multiple comparison test.

(P<0.01) was observed for R30 and R50 with simultaneous decrease in G1 in comparison to control.

Daunorubicin accumulation (Fig. 2). DB accumulation in resistant cells overexpressing P-gp is statistically significantly lower than in sensitive cells (P<0.0001). Analysis showed that cytostatic accumulation in RDB cells increased significantly following resveratrol administration at concentration of 30 μM in comparison to cells treated with cytostatic only (P<0.001).

Expression level of resistance-related proteins: P-gp, TopoIIα and TopoIIβ. Western blot method was used for the evaluation of protein expression level. Comparison of P-gp level in P and RDB cells showed high overexpression of this protein in DB-resistant line (P<0.0001). After administration of R30, R50 and combination of R30 + DB and R50 + DB, clear decrease in P-gp level was noticed in the cells of RDB line: P<0.01 for R30 and P<0.001 for others (Fig. 3A).

For RNOV line, markedly lower expression of TopoIIα and TopoIIβ was observed in comparison to P line (P<0.0001). In case of TopoIIα, resveratrol administration caused significant increase in the level of analysed proteins in comparison to the control: P<0.01 for R30, P<0.05 for R50 and P<0.001 for R30 + MTX (Fig. 3B).

On the other hand, in resistant cells the level of TopoIIβ differs significantly following cytostatic administration. The analysis showed that in comparison to the cells untreated with cytostatic during the experiment, protein level increased significantly only after R30 administration, while in comparison to the cells treated with cytostatic alone significant increase was observed for R30 (P<0.001), R30 + MTX (P<0.05) and R50 + MTX (P<0.01) in comparison with RNOV MTX. Clear difference is also visible between R50 and R50 + MTX (P<0.01) (Fig. 3C).

Immunofluorescence method was used to show high expression of P-gp in the membranes of cells from line RDB, while line RNOV is characterised by lower level of both α and β isoforms of TopoII in cell nuclei in comparison to sensitive cell line P. Localisation of individual proteins in resistant cell lines is shown in Fig. 4.

Discussion

Based on the reports regarding potential effect of resveratrol on cancer cell resistance, research was conducted on pancreatic cancer cell lines characterised by different MDR mechanisms. In case of RDB line this phenomenon is mainly associated with P-gp overexpression in the cell membrane, leading to active drug removal from cells. On the other hand, resistance of RNOV cell line is due to the lowered level and activity of TopoII, which is one of the targets for anthracyclines (33).

Research on resveratrol cytotoxicity showed that IC_{50} values are relatively high for all types of analysed cancer cells. Statistical analysis of colorimetric test and cell cycle confirmed that this compound effectively inhibits cell proliferation also in low concentrations of 30 and 50 μM. Notably, for each of the analysed cell lines this occurs in a different phase of cell cycle: S phase for sensitive cells, G1 for RDB, S and G2/M for RNOV. Cell cycle arrest caused by resveratrol in different phases was previously described, among others, as the effect on cell cycle regulating proteins e.g. cyclins, XIAP, transcription factor NF-κB, p21/waf1 (14,34-36). Inhibition in certain phase of cell cycle depends on the type of the cancer and it is associated with both cytostatic-

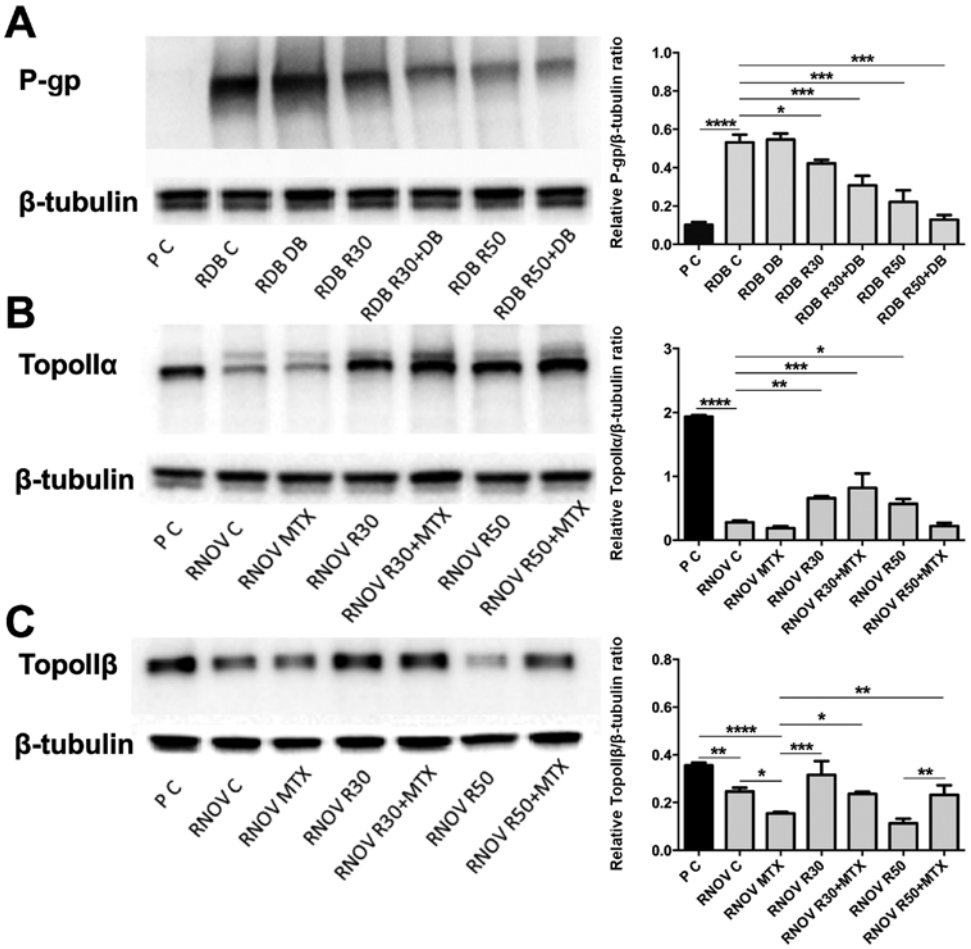

Figure 3. Expression level of P-gp, TopoIIα and TopoIIβ proteins (by western blot method). (A) P-gp in RDB cell line; (B) TopoIIα in RNOV cell line; (C) TopoIIβ in RNOV cell line. *P<0.05, **P<0.01, ***P<0.001; P C (black bars), protein level in P cell line (control P); RDB C, protein level in RDB cell line (control RDB); RNOV C, protein level in RNOV cell line (control RNOV). Analysis with the use of Dunnett's and Dunn's test.

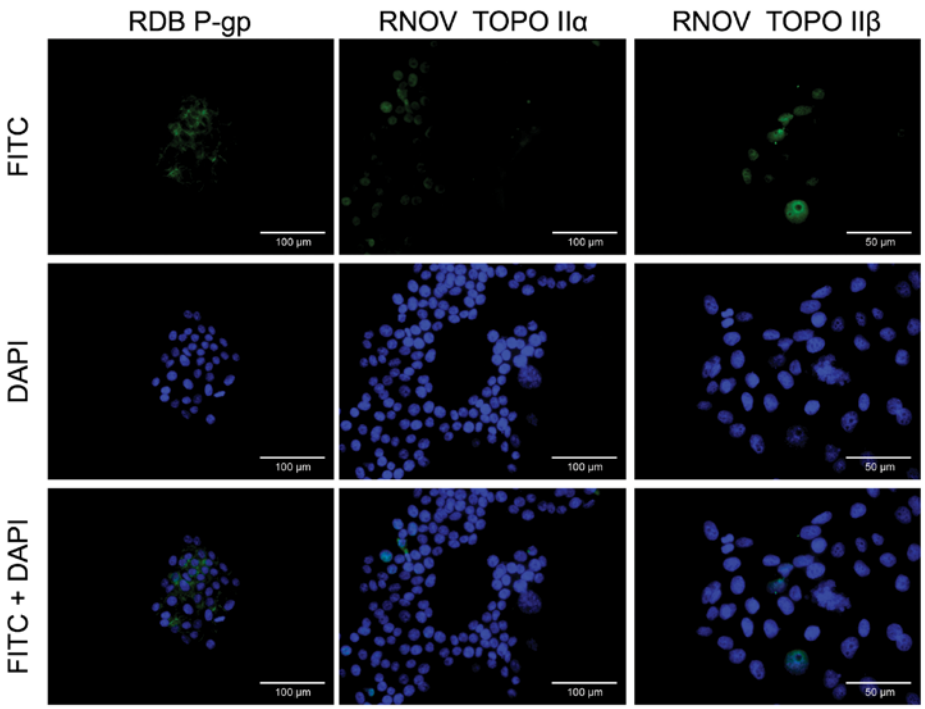

Figure 4. Immunoflurescent imaging of P-glycoprotein (P-gp) in RDB cells, as well as topoisomerase-IIα (TOPOIIα) and topoisomerase-IIβ (TOPOIIβ) in RNOV cells. FITC-conjugated antibodies were used to visualize and analyse the antigens, whereas DAPI staining was performed to visualise the nuclei. Original magnification, A and B, x200, C x400.

sensitive and resistant cells e.g. chemoresistant melanoma B16, where inhibition occurs in G1 phase and is associated with the effect on cyclin D1 and p53 protein (37,38).

The effect of polyphenols, including resveratrol, was studied in KB-C2 cells. The level of P-gp was reduced together with increased cytostatic accumulation (39,40). In our research conducted on resistant pancreatic cancer cells we showed significant increase in the accumulation of daunorubicin and mitoxantrone under the influence of the polyphenol. However, statistically significant changes were reported only in case of cells resistant to daunorubicin, over-expressing P-gp (RDB). This may indicate that the activity of this transporter is directly affected by resveratrol. The level of P-gp in the membrane of RDB cells is clearly higher than in the other two lines. After 72 h of incubation with resveratrol, expression in resistant cells decreased significantly in concentration-dependent manner. Therefore, it can be assumed that in the studied cells resveratrol significantly reduces the level of P-gp at post-translational modification stage i.e. proteome or metabolome. Other polyphenols, mainly quercetin, reduce not only P-gp level, but also expression of its encoding gene, *ABCB1*, including cells of human pancreatic and stomach cancer (41,42).

The research on the effect of resveratrol on TopoII expression level is relatively new. It is known that in glioblastoma U87 cell line resveratrol may unspecifically interact both with DNA and TopoII, which was shown with the use of molecular docking mechanisms. In the cells expressing topoisomerases on normal or increased level resveratrol attaches to the cleavable TopoII-DNA complex thus forming a network of hydrogen bonds and causing its stabilisation (43), thus, it acts similarly to cytostatics. Moreover, its effect on the activity of TopoIIα was confirmed, which results in the loss of capability to disentangle and remove DNA knots and supercoils. Breakage of double-stranded DNA may be caused additionally by prolongation of S-phase cell cycle as a result of histone H2AX phosphorylation (44). The enhancement of doxorubicin activity as a poison for TopoII was recently described for colon cancer cell line, wherein used doses of resveratrol were much higher than in our studies (45). In the cells with reduced expression of TopoII daunorubicin has much lower effectiveness due to the small number of cleavable complexes. By increasing the level of TopoII, mainly its α isoform, resveratrol sensitises cells to cytostatics. S-phase cell cycle inhibition was observed for studied RNOV cells, which are characterised by reduced expression of TopoII, and in P cell line. Upon resveratrol administration in RNOV cell line significant increase in the level of expression of both TopoII isomers was observed, in particular, at a lower concentration of the polyphenol it suggests the effect of resveratrol on so-called atypical mechanism of resistance.

In conclusion, resveratrol is a compound that inhibits the cell cycle in studied human pancreatic cancer cell lines, both sensitive and resistant to cytostatics. Moreover, it affects MDR phenomenon caused by both P-gp overexpression and atypical cases of resistance, such as reduced level of TopoII. It proves that resveratrol has not only direct anticancer properties, but it may also be use in sensitising cancer cells to chemotherapy by multidirectional action on resistance-causing mechanisms.

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References

1. Liu BL, Zhang X, Zhang W and Zhen HN: New enlightenment of French Paradox: Resveratrol's potential for cancer chemoprevention and anti-cancer therapy. Cancer Biol Ther 6: 1833-1836, 2007.
2. Meng C, Liu JL and Du AL: Cardioprotective effect of resveratrol on atherogenic diet-fed rats. Int J Clin Exp Pathol 7: 7899-7906, 2014.
3. Zheng JP, Ju D, Jiang H, Shen J, Yang M and Li L: Resveratrol induces p53 and suppresses myocardin-mediated vascular smooth muscle cell differentiation. Toxicol Lett 199: 115-122, 2010.
4. Smoliga JM, Baur JA and Hausenblas HA: Resveratrol and health: a comprehensive review of human clinical trials. Mol Nutr Food Res 55: 1129-1141, 2011.
5. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S and Takada Y: Role of resveratrol in prevention and therapy of cancer: Preclinical and clinical studies. Anticancer Res 24 (5A): 2783-2840, 2004.
6. Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR and Kim AL: Resveratrol: A review of preclinical studies for human cancer prevention. Toxicol Appl Pharmacol 224: 274-283, 2007.
7. Park EJ and Pezzuto JM: The pharmacology of resveratrol in animals and humans. Biochim Biophys Acta 1852: 1071-1113, 2015.
8. Novelle MG, Wahl D, Diéguez C, Bernier M and de Cabo R: Resveratrol supplementation: Where are we now and where should we go? Ageing Res Rev 21: 1-15, 2015.
9. Dhar S, Kumar A, Li K, Tzivion G and Levenson AS: Resveratrol regulates PTEN/Akt pathway through inhibition of MTA1/HDAC unit of the NuRD complex in prostate cancer. Biochim Biophys Acta 1853: 265-275, 2015.
10. Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, *et al*: Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275: 218-220, 1997.
11. Zhang J, Ma K, Qi T, Wei X, Zhang Q, Li G and Chiu JF: P62 regulates resveratrol-mediated Fas/Cav-1 complex formation and transition from autophagy to apoptosis. Oncotarget 6: 789-801, 2015.
12. Wu XP, Xiong M, Xu CS, Duan LN, Dong YQ, Luo Y, Niu TH and Lu CR: Resveratrol induces apoptosis of human chronic myelogenous leukemia cells in vitro through p38 and JNK-regulated H2AX phosphorylation. Acta Pharmacol Sin 36: 353-361, 2015.
13. Fulda S and Debatin KM: Sensitization for anticancer drug-induced apoptosis by the chemopreventive agent resveratrol. Oncogene 23: 6702-6711, 2004.
14. Gupta SC, Kannappan R, Reuter S, Kim JH and Aggarwal BB: Chemosensitization of tumors by resveratrol. Ann NY Acad Sci 1215: 150-160, 2011.
15. Türedi S, Yuluğ E, Alver A, Kutlu Ö and Kahraman C: Effects of resveratrol on doxorubicin induced testicular damage in rats. Exp Toxicol Pathol 67: 229-235, 2014.
16. Zhang L, Guo X, Xie W, Li Y, Ma M, Yuan T and Luo B: Resveratrol exerts an anti-apoptotic effect on human bronchial epithelial cells undergoing cigarette smoke exposure. Mol Med Rep 11: 1752-1758, 2015.
17. Gottesman MM and Ling V: The molecular basis of multidrug resistance in cancer: The early years of P-glycoprotein research. FEBS Lett 580: 998-1009, 2006.
18. Colabufo NA, Contino M, Berardi F, Perrone R, Panaro MA, Cianciulli A, Mitolo V, Azzariti A, Quatrale A and Paradiso A: A new generation of MDR modulating agents with dual activity: P-gp inhibitor and iNOS inducer agents. Toxicol In Vitro 25: 222-230, 2011.

19. Ullah MF: Cancer multidrug resistance (MDR): A major impediment to effective chemotherapy. Asian Pac J Cancer Prev 9: 1-6, 2008.
20. Baguley BC: Multiple drug resistance mechanisms in cancer. Mol Biotechnol 46: 308-316, 2010.
21. Lage H: An overview of cancer multidrug resistance: A still unsolved problem. Cell Mol Life Sci 65: 3145-3167, 2008.
22. Nitiss JL: DNA topoisomerase II and its growing repertoire of biological functions. Nat Rev Cancer 9: 327-337, 2009.
23. Burgess DJ, Doles J, Zender L, Xue W, Ma B, McCombie WR, Hannon GJ, Lowe SW and Hemann MT: Topoisomerase levels determine chemotherapy response in vitro and in vivo. Proc Natl Acad Sci USA 105: 9053-9058, 2008.
24. Järvinen TA and Liu ET: Topoisomerase IIalpha gene (TOP2A) amplification and deletion in cancer - more common than anticipated. Cytopathology 14: 309-313, 2003.
25. Carrato A, Falcone A, Ducreux M, Valle JW, Parnaby A, Djazouli K, Alnwick-Allu K, Hutchings A, Palaska C and Parthenaki I: A Systematic review of the burden of pancreatic cancer in europe: Real-world impact on survival, quality of life and costs. J Gastrointest Cancer 46: 201-211, 2015.
26. Boyle J, Czito B, Willett C and Palta M: Adjuvant radiation therapy for pancreatic cancer: A review of the old and the new. J Gastrointest Oncol 6: 436-444, 2015.
27. Xu Q, Zong L, Chen X, Jiang Z, Nan L, Li J, Duan W, Lei J, Zhang L, Ma J, *et al*: Resveratrol in the treatment of pancreatic cancer. Ann NY Acad Sci 1348: 10-19, 2015.
28. Shankar S, Nall D, Tang SN, Meeker D, Passarini J, Sharma J and Srivastava RK: Resveratrol inhibits pancreatic cancer stem cell characteristics in human and KrasG12D transgenic mice by inhibiting pluripotency maintaining factors and epithelial-mesenchymal transition. PLoS One 6: e16530, 2011.
29. Lage H, Jordan A, Scholz R and Dietel M: Thermosensitivity of multidrug-resistant human gastric and pancreatic carcinoma cells. Int J Hyperthermia 16: 291-303, 2000.
30. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR: New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107-1112, 1990.
31. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
32. Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354, 1979.
33. Lage H and Dietel M: Multiple mechanisms confer different drug-resistant phenotypes in pancreatic carcinoma cells. J Cancer Res Clin Oncol 128: 349-357, 2002.
34. Harikumar KB and Aggarwal BB: Resveratrol: A multitargeted agent for age-associated chronic diseases. Cell Cycle 7: 1020-1035, 2008.
35. Cui J, Sun R, Yu Y, Gou S, Zhao G and Wang C: Antiproliferative effect of resveratrol in pancreatic cancer cells. Phytother Res 24: 1637-1644, 2010.
36. Wang G, Dai F, Yu K, Jia Z, Zhang A, Huang Q, Kang C, Jiang H and Pu P: Resveratrol inhibits glioma cell growth via targeting oncogenic microRNAs and multiple signaling pathways. Int J Oncol 46: 1739-1747, 2015.
37. Gatouillat G, Balasse E, Joseph-Pietras D, Morjani H and Madoulet C: Resveratrol induces cell-cycle disruption and apoptosis in chemoresistant B16 melanoma. J Cell Biochem 110: 893-902, 2010.
38. Chin YT, Hsieh MT, Yang SH, Tsai PW, Wang SH, Wang CC, Lee YS, Cheng GY, HuangFu WC, London D, *et al*: Anti-proliferative and gene expression actions of resveratrol in breast cancer cells in vitro. Oncotarget 5: 12891-12907, 2014.
39. Nabekura T, Kamiyama S and Kitagawa S: Effects of dietary chemopreventive phytochemicals on P-glycoprotein function. Biochem Biophys Res Commun 327: 866-870, 2005.
40. Quan F, Pan C, Ma Q, Zhang S and Yan L: Reversal effect of resveratrol on multidrug resistance in KBv200 cell line. Biomed Pharmacother 62: 622-629, 2008.
41. Borska S, Sopel M, Chmielewska M, Zabel M and Dziegiel P: Quercetin as a potential modulator of P-glycoprotein expression and function in cells of human pancreatic carcinoma line resistant to daunorubicin. Molecules 15: 857-870, 2010.
42. Borska S, Chmielewska M, Wysocka T, Drag-Zalesinska M, Zabel M and Dziegiel P: In vitro effect of quercetin on human gastric carcinoma: Targeting cancer cells death and MDR. Food Chem Toxicol 50: 3375-3383, 2012.
43. Leone S, Basso E, Polticelli F and Cozzi R: Resveratrol acts as a topoisomerase II poison in human glioma cells. Int J Cancer 131: E173-E178, 2012.
44. Leone S, Cornetta T, Basso E and Cozzi R: Resveratrol induces DNA double-strand breaks through human topoisomerase II interaction. Cancer Lett 295: 167-172, 2010.
45. Schroeter A and Marko D: Resveratrol modulates the topoisomerase inhibitory potential of doxorubicin in human colon carcinoma cells. Molecules 19: 20054-20072, 2014.