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Intracellular glutathione level and efflux in human melanoma and cervical cancer cells differing in doxorubicin resistance

Poziom wewnątrzkomórkowego glutationu oraz jego wypływ w komórkach ludzkiego czerniaka i raka szyjki macicy o zróżnicowanej oporności na doksorubicynę

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Drug resistance continues to be a major problem in cancer treatment. Occurrence of this phenomenon is often associated with altered levels of glutathione (GSH) and GSH-related enzymes. The aim of the study was to evaluate the possible involvement of GSH and GSH-related enzymes in doxorubicin (DOX) resistance in two types of cancer cells of different etiology, from both parental and DOX-resistant sublines.

Materials and methods: The human melanoma (ME18 and ME18/R) and cervical cancer cells (HeLa and KB-V1) were tested in terms of their DOX sensitivity (EZ4U test), GSH level (HPLC) and its efflux (spectrofluorometrically). The effects of inhibition of the GSH-related enzymes γ-glutamylcysteine synthetase (γ-GCS) and glutathione S-transferase (GST) were also evaluated.

Results: Exposure to DOX caused an increase of GSH levels in all tested cells except for HeLa cells. However, depletion of GSH did not have a significant influence on the sensitivity of the cells to DOX. Inhibition of the activity of GST also did not have a major effect on DOX sensitivity, although it caused changes of the GSH content. Our attempts to use the spectrofluorometric method for measurements of GSH efflux were not successful. It could be suggested that in ME18 and HeLa cells treated with DOX, GSH efflux does occur.

Discussion: The obtained results seem to refute the hypothesis of a central role of GSH in DOX resistance of the tested cells. Despite observations of different effects related to GSH, they do not seem to be essential in terms of DOX resistance. The mechanisms underlying DOX resistance are highly cell-specific.

Key words: drug resistance • doxorubicin • glutathione • melanoma • cervical cancer • ethacrynic acid • buthionine sulfoximine

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Abbreviations: **BSO** – buthionine sulfoximine, **DOX** – doxorubicin, **DTNB** – 5,5'-dithiobis-(2-nitrobenzoic acid), Ellman's reagent, **EA** – ethacrynic acid, **FBS** – fetal bovine serum, **γ-GCS** – γ-glutamylcysteine synthetase, **GSH** – glutathione, reduced form, **GSSG** – glutathione, oxidized form, **GST** – glutathione S-transferase, **IC_{50}** – half maximal inhibitory concentration, **MEM** – minimal essential medium, **MRP** – multidrug resistance-related protein, **PBS** – phosphate buffered saline, **RF** – resistance factor = IC_{50} for resistant cells/IC_{50} for sensitive cells, **ROS** – reactive oxygen species.

Introduction

The anthracycline antibiotic doxorubicin (DOX) is one of the most widely used antineoplastic agents, displaying clinical activity against a broad range of cancers, including lymphoma, multiple myeloma, lung, ovarian, gastric, thyroid, breast, sarcoma, and pediatric cancers. Its antitumor activity consists of two major mechanisms: intercalation into DNA resulting in inhibition of DNA synthesis or topoisomerase II activity; and generation of free radicals, leading to DNA and cell membrane damage [6,7,21,37,47]. Development of resistance to DOX continues to be a main problem in cancer treatment, dramatically limiting the number of available therapeutic options.

Glutathione (GSH) is one of the major factors contributing to drug resistance by reacting with drugs, reducing ROS, preventing damage of proteins or DNA, and by involvement in DNA repair processes [36,51]. The physiological properties of glutathione are based on continuous processes of reversible conversion of the reduced form of GSH into the oxidized GSSG. The equilibrium of GSH/GSSG is essential for the cells to maintain the redox balance and to survive in the conditions of oxidative stress.

High levels of GSH are observed in various types of cancer cells, and make the neoplastic tissues more resistant to chemotherapy [9,14,56]. It was found that the content of GSH in some tumor cells is typically associated with elevated levels of GSH-related enzymes, such as glutamylcysteine synthetase (γ-GCS), the key enzyme in GSH biosynthesis [3,24,39, 57]. Therefore, the GSH system has been considered as a possible target to overcome drug resistance of cancer cells [15]. The main research in this field has been focused at depleting GSH by using specific inhibition of γ-GCS. Buthionine sulfoximine (BSO) is the most popular agent for this purpose [4,13,22]. Studies involving a number of human cancer cells have shown that inhibition of γ-GCS sensitizes them to treatment with platinum compounds, alkylating agents, anthracyclines and arsenic trioxide [3,20,30,41]. *In vitro* studies have proved that even partial inhibition of GSH synthesis can increase the efficacy of treatment [30].

A major function of GSH is the detoxification of xenobiotics and some endogenous compounds. These substances are electrophiles and form conjugates with GSH, either spontaneously or enzymatically, in reactions catalyzed by GSH S-transferases (GSTs) [54]. Human GSTs are divided into two family members: cytosolic and membrane-bound microsomal [23,52]. It was found that overexpression of GSTs combined with high GSH levels can affect drug resistance by detoxification of chemotherapeutics and reducing their effectiveness [35]. Inhibition of GST activity is therefore considered as a potentially useful approach to modulate resistance to certain antineoplastic agents, such as alkylating agents and anthracyclines. Most research has used EA as a nonselective inhibitor of GST [43]. The results of some studies indicate that GST activity may also be required to remove conjugates of GSH from the cell via specific transporters, including multidrug resistance protein (MRP) dependent pathways [8,32,42]. MRP1 appears to transport drugs conjugated to GSH and also unmodified cytostatic agents in the presence of GSH [40]

The aim of this study was to characterize and compare cancer cells of different etiology: human melanoma cells (ME18 and ME18/R) and cervical cancer cells (HeLa and KB-V1), from both parental and DOX-resistant corresponding sublines, in terms of their DOX sensitivity, GSH level and its efflux. The effects of inhibition of activity of two GSH-related enzymes, γ-GCS and GST, were also evaluated.

Materials

DOX (hydrochloride) ≥98%; BSO; antimycotic-antibiotic; GST; dithiothreitol, sulfosalicylic acid; Tris-buffer (Sigma); EA; MCB (Fluka); DMSO (LabScan); EZ4U Cell Proliferation Assay kit (Biomedica, Poland); MEM and FBS (Lonza); 0.5% trypsin-EDTA (Gibco); calcium and magnesium ionfree PBS (IITD, Wrocław); orthophosphoric acid, sodium hydroxide and disodium hydrophosphate salt (POCh); DTNB (Merck); GSH (BDH Biochemicals).

Cell lines

- human melanoma cells: ME18 and ME18/R.
  ME18 cells were a gift from the Institute of Oncology, Warsaw, Poland.
  ME18/R is a unique ME18 subline resistant to DOX, developed at the National Medicines Institute [1].

- human cervix carcinoma cells: HeLa and KB-V1. Both cell lines were obtained from the ATCC collection.
  KB-V1 is a multidrug-resistant subclone derived from KB-3-1, which itself is a derivative of HeLa cells. The KB-V1 cell line was developed through selection with vinblastine, but it also expresses resistance to colchicine and DOX [50].

Methods

Culture conditions

The cells were cultured in MEM medium supplemented with 10% FBS and 1% antimycotic-antibiotic at 37°C in 5% CO_2 atmosphere.

Treatment protocol

Effects of DOX treatment were evaluated in several combination settings. Combination of treatment with DOX after BSO pretreatment was designed to investigate the effect of GSH depletion, whereas combination of DOX and EA was designed to examine the result of inhibition of GST activity.

24-hour cell cultures were prepared as follows:

- for cytotoxicity testing – untreated cells; cells treated with various concentrations of DOX for 24 hours; cells pretreated with BSO for 24 hours and then treated with DOX for 24 hours; cells co-treated with DOX and EA for 24 hours,

- for HPLC analysis – untreated cells; cells treated with DOX, BSO or EA for 24 hours; cells pretreated with BSO for 24 hours and then treated with DOX for 24 hours; cells co-treated with DOX and EA for 24 hours.

Before the study, IC_{50} values after 24 hours of treatment were determined for DOX, BSO and EA for each cell line.

In the cytotoxicity test, the cells were treated with various DOX concentrations for evaluation of the IC_{50} values. DOX concentrations selected to be used throughout the study were as follows: 0.9 µM for ME18, 34.0 µM for ME18/R, 9.0 µM for HeLa and 69.0 µM for KB-V1. To show the potential differences between the responses of the sensitive and the DOX-resistant cells, they were also exposed to a lower DOX concentration, which was half the concentration used for the sensitive sublines.

BSO was used at its highest non-cytotoxic concentration, i.e. 50 µM for ME18, HeLa and KB-V1 and 100 µM for ME18/R. The cells were preincubated for 24 hours with BSO before exposure to DOX, as suggested by Griffith [22] and Sawyer and Bonner [48].

EA was used at its highest non-cytotoxic concentration, i.e. 100 µM for ME18 and ME18/R and 25 µM for HeLa and KB-V1. It was, in turn, used with DOX simultaneously during the 24-hour treatment.

Cytotoxicity test

Cells were plated in 96-well plates in the amount of $3x10^4$ cells/100 µl/well (7 wells per experimental condition). After 24 hours, the cells were exposed to various concentrations of DOX, BSO or EA for 24 hours. The final solutions of DOX, BSO and EA were prepared in growth medium and added to the wells accordingly. After incubation, the cells were examined microscopically and cell viability was assessed using the EZ4U Cell Proliferation Assay kit. Previously, the EZ4U test has been validated in our laboratory and it has been proven to give comparable results to the reference MTT test [29]. The test was performed according to the test manufacturer's protocol. The EZ4U test consists in transformation of tetrazolium salts into water-soluble, intensely colored formazan derivatives. The reaction is catalyzed by succinate dehydrogenase, present in living cells. Spectrophotometric measurement allows the evaluation of cell viability and proliferation, which is proportional to color intensity. After measurements, the inhibitory 50% concentration (IC_{50}) was determined. The resistance factor (RF) was obtained by dividing the IC_{50} value of the resistant cell line by the IC_{50} value of the non-resistant cell line and represents the drug's efficacy against drug-resistant cell lines vs. the corresponding sensitive cell lines. After exposure to resistance modifiers or inhibitors, RF can be reduced to values approaching unity, which is a sign of chemosensitization of the cells.

Preparation of cell extracts for HPLC analysis

24-hour cell cultures were used for HPLC determination. The cells were trypsinized and resuspended in growth medium. The cell density was evaluated using a Coulter Z2 Counter. The suspensions were centrifuged at 1000 rpm for 5 minutes. The cells were extracted with 1 ml of 4.5% sulfosalicylic acid for 5 minutes at room temperature. Precipitates were removed by centrifugation at 10 000 rpm for 5 minutes. The supernatants were collected and used for further analysis.

Derivatization

Derivatization using DTNB was primarily performed to enable chromatographic separation. DTNB has been used in many analytical methods to quantify sulfhydryl compounds. This step was performed according to Katrusiak et al. [26], with further modifications [33].

The reaction mixture for GSH determination consisted of 50 µl of the tested supernatant, 90 µl of 0.1M NaOH and 140 µl of 2.5 mg/ml DTNB solution in 0.3 M disodium hydrophosphate solution, pH 8.5. After 5 minutes the sample was analyzed by HPLC.

The reaction mixture for total glutathione determination (GSH and GSSG) consisted of 50 µl of the tested supernatant, 90 µl of 0.1 M NaOH and 10 µl of 3 mg/ml dithio-

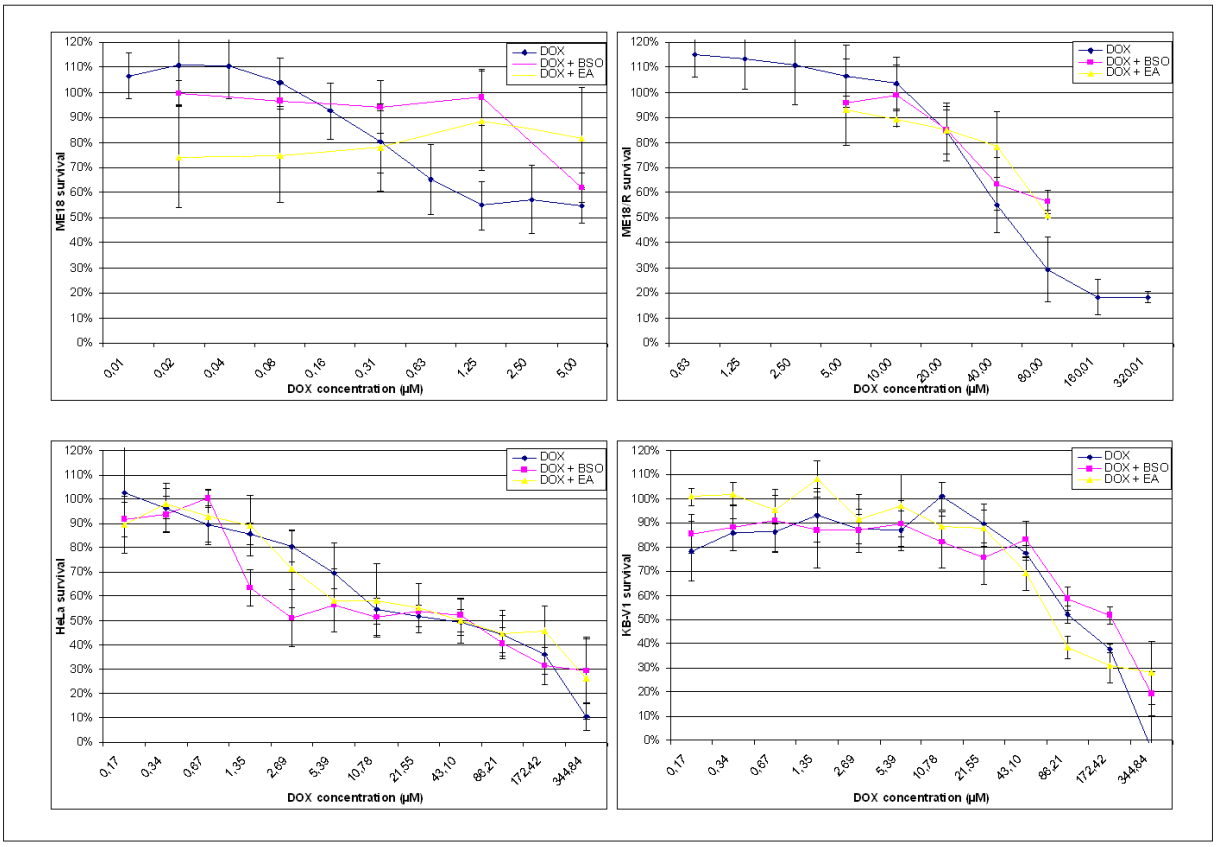

Fig. 1. Cytotoxicity of DOX alone and after preincubation with BSO or co-incubation with EA for 24 hours in: ME18 and ME18/R; HeLa and KB-V1 cells. BSO was used at the concentration of 50 μM for ME18, HeLa, KB-V1 and 100 μM for ME18/R cells. EA was used at the concentration of 100 μM for ME18, ME18/R and 25 μM for HeLa, KB-V1.
Results are the mean values obtained from 2 independent experiments, each performed in triplicate

threitol in 0.3 M disodium hydrophosphate solution, pH 8.5. After 5 minutes, 150 μl of 2.5 mg/ml DTNB solution in 0.3 M disodium hydrophosphate solution, pH 8.5, was added. After 5 minutes the sample was analyzed by HPLC.

HPLC

The analysis was performed according to Katrusiak et al. [26], with further modifications [33]. HPLC was accomplished using isocratic elution on a Supelcosil LC-18DB 5 μm 250 x 4.6 column at 40°C with detection of UV absorbance at 330 nm. The mobile phase consisted of methanol and 0.1 M sodium phosphate buffer, pH 3.8, at a flow rate of 1 ml/min. Increase of methanol content during the gradient was used to elute excess DTNB reagent. Then it was decreased to allow re-equilibration of the column before injection of the next sample.

The results of HPLC determination were presented as μg of GSH or GSSG per 10^6 cells.

Spectrofluorometric determination of GSH efflux

Evaluation was performed according to Kamencic et al. [25]. The method was previously used for measurements of GSH in cell lysates or tissue homogenates. In the study, we attempted to use the method for direct measurements of GSH released from cells to the growth medium.

After 24 hours of incubation, growth media from cultures were collected and centrifuged at 5000 rpm for 5 minutes at 4°C to discard any dead cells. Supernatants were kept on ice and used for measurements immediately. Simultaneously, the cells were trypsinized and counted using a Coulter Z2 Counter.

MCB and GST were added to the tested supernatants to a final concentration of 100 μM and 1 U/ml, respectively. The reaction mixtures were distributed to 96-well microtiter plates and allowed to incubate at room temperature for 30 minutes. The GSH-MCB adduct was measured with excitation at 355 nm and emission measured at 460 nm.

All reagents were kept on ice before use. Positive controls (GSH standard dissolved in 50 mM Tris buffer, pH 7.4, and further diluted in MEM medium) and negative controls (MEM medium – without GSH) were included in each test. The tested reaction mixtures were spiked with 10 μM GSH standard to raise the measured fluorescence above the limit of quantification (LOQ) of the method.

The amount of GSH released to the medium was calculated as the fluorescence intensity per 10^6 cells. The results were presented as the relative amount of GSH released from the cells treated with DOX compared to the amount of GSH released from control (untreated) cells.

Statistical analysis

The results were expressed as the mean ±SD of at least two independent experiments, each performed in duplicate or triplicate. The Medistat System (a microcomputer statistical system for medicinal purposes, version 2.1; 1992) was used to check the statistical significance of the obtained results. Student's two-tailed, unpaired t-test was used, and values of $p<0.05$ were considered to be significant.

Results

Cytotoxicity of DOX

The sensitivity of four cell lines – ME18, HeLa and corresponding DOX-resistant ME18/R and KB-V1 – was examined.

Cell survival is presented as the percentage of living cells in culture exposed to DOX compared to the untreated culture in Figure 1.

The IC_{50} values of DOX after 24 hours of incubation were estimated as 1.25 µM for ME18, 40.0 µM for ME18/R, 10.8 µM for HeLa and 86.2 µM for KB-V1 cells. The IC_{50} values of DOX were higher in cervical carcinoma cells compared to melanoma cells. The RF value for melanoma cells (RF=32) was, however, 4-fold higher than for cervical carcinoma cells (RF=8). Microscopic evaluation confirmed the cytotoxic effect of DOX against tested cells (Figure 2).

Intracellular glutathione level

The obtained results are presented as GSH content in µg per 10^6 cells (Figure 3). They indicate that there are significant differences in native intracellular GSH levels between the tested cells. The native GSH level was 2.1 µg/10^6 cells in untreated ME18 cells and 3.5 µg/10^6 cells in ME18/R cells. DOX-resistant melanoma cells synthesized up to 64% more GSH than the corresponding sensitive line.

GSH content in both melanoma cell lines was positively correlated with increasing DOX concentration. After treatment with DOX used at concentrations of 0.9 µM for ME18 and 34.0 µM for ME18/R, GSH level increased to 212% in ME18 cells and to 140% in ME18/R. In both melanoma cell lines the final GSH content was apparently similar, 4.5 µg/10^6 cells and 4.9 µg/10^6 cells, respectively. It was also observed that DOX treatment resulted in the increase of GSSG levels to comparable levels in both cells, i.e., to 4-fold higher in the ME18 cells and 4.5-fold higher in ME18/R cells (Figure 3).

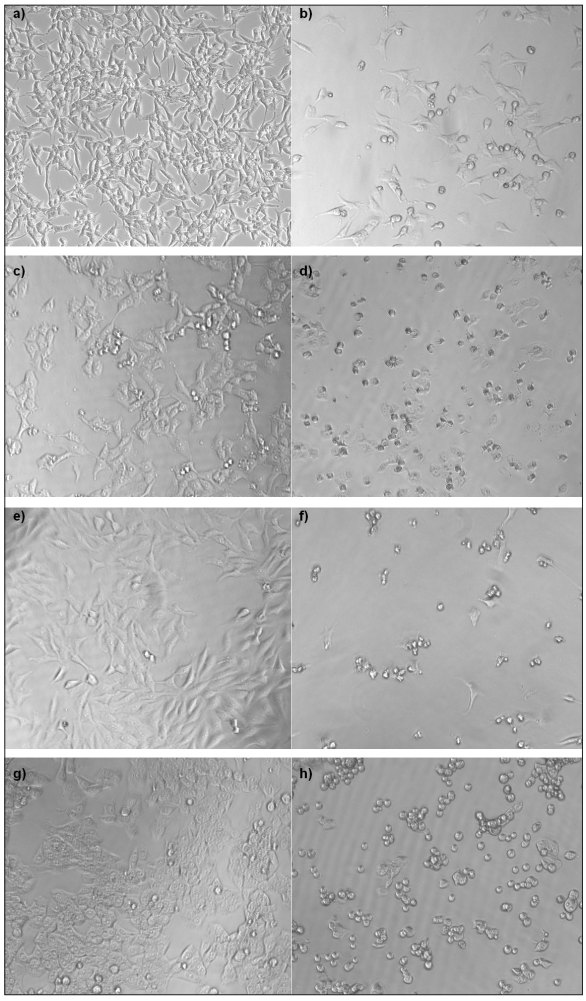

Fig. 2. a-b) ME18, c-d) ME18/R, e-f) HeLa and g-h) KB-V1 cells in untreated cultures and after 24 hr exposure do DOX. DOX was used at IC50 concentrations, i.e. 1,25 µM for ME18 (b); 40,0 µM for ME18/R (d); 10,8 µM for HeLa (f); 86,2 µM for KB-V1 (h)

In the case of HeLa and KB-V1 cells, the native GSH content in HeLa cells was 171% higher than in KB-V1 cells, and it was 31 µg/10^6 cells and 11.4 µg/10^6 cells, respectively (Figure 3). GSH level in HeLa was the highest among all tested cells.

Effects caused by exposure to DOX were also different in cervical carcinoma cells. In HeLa cells, treatment with 9.0 µM DOX caused a decrease of the GSH content by 33%, with simultaneous 20-fold elevation of GSSG level (Figure 3). The opposite correlation was observed in KB-V1 cells, in which exposure to 69.0 µM DOX increased the GSH level up to 274%. At the same time, GSSG level changed insignificantly as compared to the untreated cells (Figure 3).

Glutathione efflux

Data obtained from measurements using the GSH standard were used to make the standard curve, plotting GSH concentration on the X axis and the measured fluores-

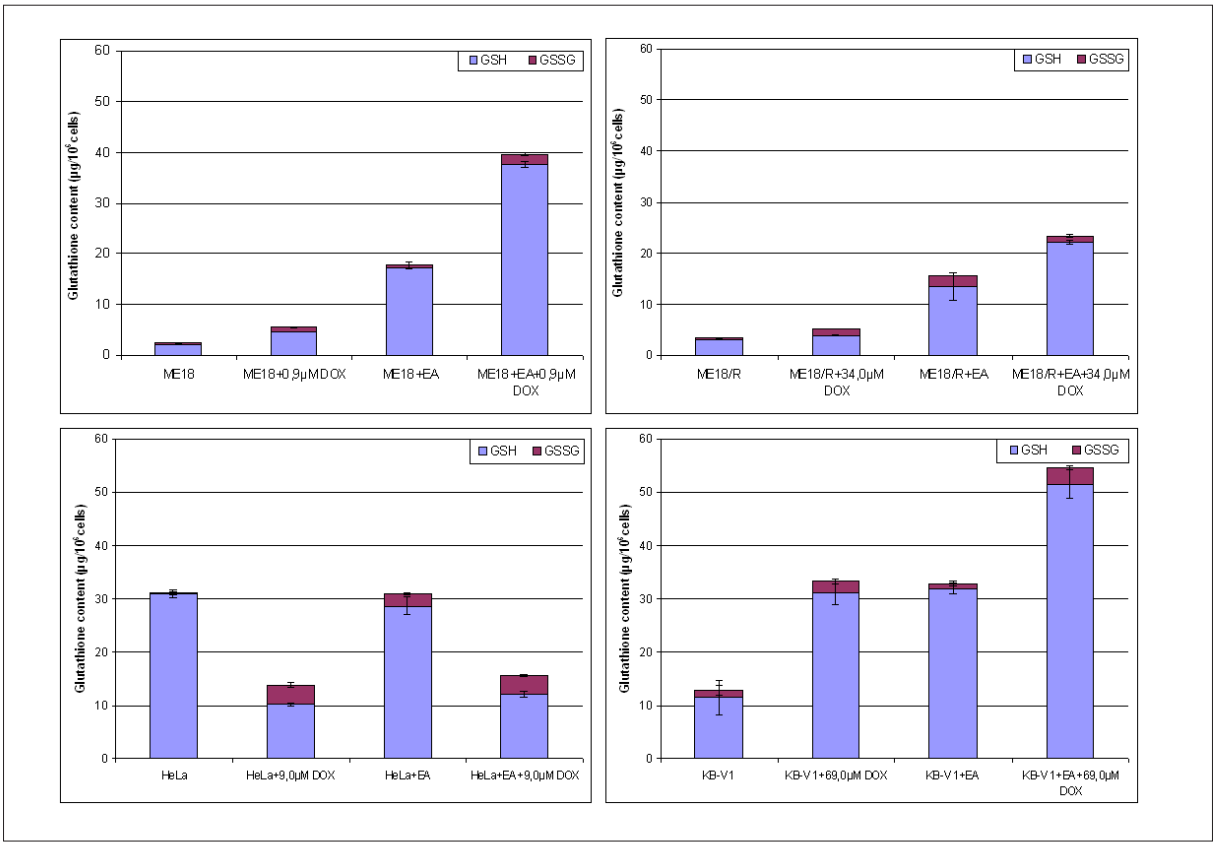

Fig. 3. GSH and GSSG content in ME18 and ME18/R; HeLa and KB-V1 cells; after 24 hr treatment with DOX or 24 hr co-treatment with DOX and EA. DOX was used at the concentrations of 0,9 µM for ME18; 34,0 µM for ME18/R; 9,0 µM for HeLa; 69,0 µM for KB-V1. EA was used at the concentration of 100 µM for ME18, ME18/R and 25 µM for HeLa, KB-V1. Results are the mean values obtained from 2 or 3 independent experiments, each performed in triplicate

cence intensity on the Y axis (Figure 4). Based on the calculations, a high value for the coefficient of determination (R^2 = 0.9971) was achieved, confirming the linearity of the method. LOQ was also calculated (1.56 µM), and it was consistent with LOQ obtained by Kamencic et al. [25].

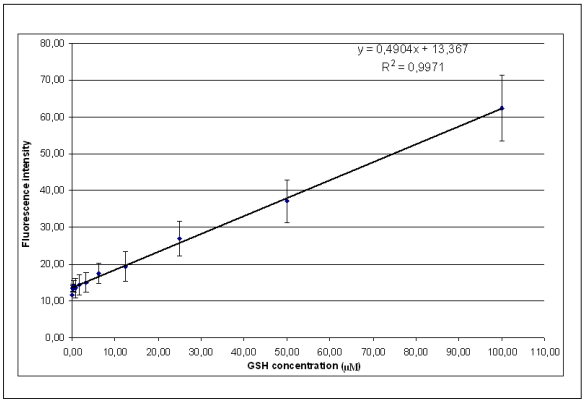

Fig. 4. Linear correlation between GSH concentration and fluorescence intensity. Results are the mean values obtained from 8 independent measurements performed using GSH standard. Limit of detection (LOD) was 0,78 µM GSH and limit of quantification (LOQ) was 1,56 µM GSH

To minimize the predicted error for low-level measurements of tested samples, reaction mixtures were spiked with 10 µM of GSH standard in order to raise their fluorescence (to be within the scope of quantification). Obtained results are presented in Figure 5.

The results obtained for ME18 cells indicate that treatment of these cells with DOX results in increase of GSH efflux to the culture medium. Exposure to DOX at the concentration of 0.45 µM resulted in a 57% increase of GSH level in the medium, while DOX at 0.9 µM caused a 150% increase. The observed GSH efflux was dose-dependent and statistically significant (Figure 5). Treatment of ME18/R with DOX at any of the tested concentrations did not significantly influence the level of GSH efflux (high deviation between the experiments prevents data interpretation).

Effects of DOX treatment observed in HeLa cells were similar to those observed in ME18 cells. DOX used at the concentrations of 4.5 µM and 9.0 µM caused 54% and 85% increase of GSH efflux to the culture medium, respectively. The observed correlation was statistically significant, but not dose-dependent. In KB-V1 cells the level of GSH released to the medium remained insignificantly changed after exposure to 4.5 µM and 69.0 µM DOX (Figure 5).

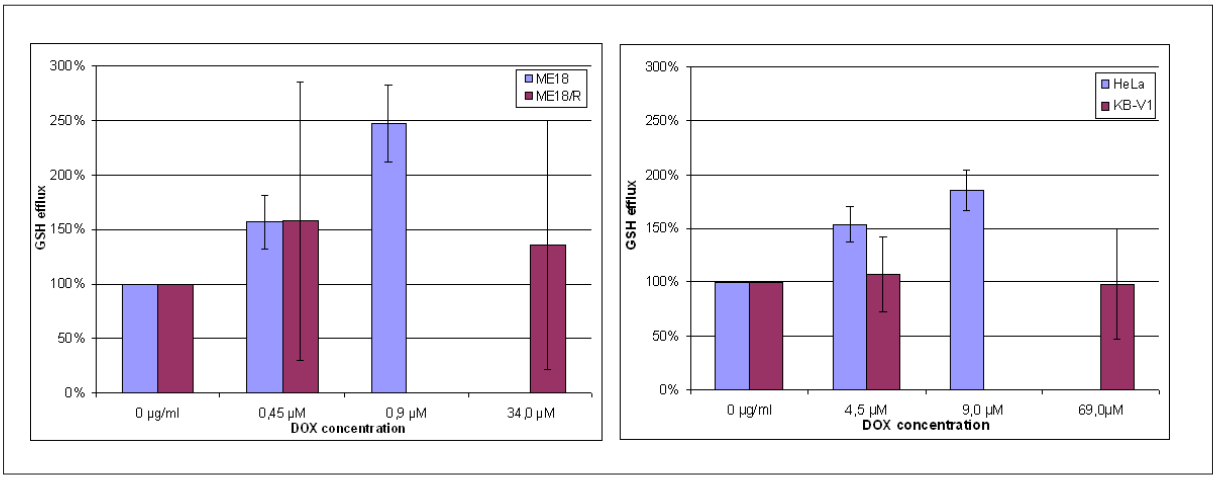

Fig. 5. GSH efflux from ME18 and ME18/R cells; HeLa and KB-V1 cells; after 24 hr exposure to DOX. DOX was used at the concentrations of 0,45 µM and 0,9 µM for ME18; 0,45 µM and 34,0 µM for ME18/R; 4,5 µM and 9,0 µM for HeLa; 4,5 µM and 69,0 µM for KB-V1. Results are the mean values obtained from 3 independent experiments, each performed in triplicate

Depletion of GSH by BSO

BSO irreversibly inhibits activity of γ-GCS, an enzyme responsible for GSH synthesis. As was expected and confirmed by HPLC assay, 24-hour BSO treatment caused complete depletion of GSH to non-detectable levels in all tested cells (data not shown).

The sensitivity of the tested cells pretreated with BSO and exposed to various concentrations of DOX was examined in the cytotoxicity test (Figure 1). It was found that pretreatment with BSO did not change the IC_{50} values of DOX in any of the tested cells except for HeLa cells, which were slightly more sensitive to DOX.

Statistical analysis confirmed that depletion of GSH had no significant influence on the sensitivity of the cells to DOX after 24-hour exposure.

Effects of GST inhibition

EA is a substrate analogue for GST and it competitively forms conjugates with GSH [44]. Its use is supposed to result in inhibition of conjugation between GSH and DOX, consequently stopping further detoxification of the drug or its transport outside the cells. As shown in Figure 3, treatment with EA resulted in GSH accumulation in the tested cells.

EA exposure caused an 8-fold increase of GSH in ME18 cells and 4-fold in ME18/R cells to the levels of 17 µg/10^6 cells and 13.5 µg/10^6 cells, respectively.

After co-incubation of melanoma cells with EA and DOX, detected levels of GSH were significantly higher as compared to the cultures kept with EA alone. At the same time, the GSH level in ME18 cells was higher than in the ME18/R line. It was also found that co-incubation of ME18 cells with EA and DOX caused a 2.7-fold increase of the GSSG level as compared to the cells treated with EA alone, while in ME18/R the GSSG level decreased 2-fold (Figure 3).

A similar correlation was also observed in KB-V1 cells, in which exposure to EA alone resulted in a 3-fold increase of GSH level up to 32 µg/10^6 cells. After co-treatment with DOX and EA, the GSH level increased to 51.5 µg/10^6 cells (161%). Simultaneously, the GSSG level was 3-fold increased (Figure 3).

Only in HeLa cells, EA did not seem to affect the GSH level (Figure 3). After co-treatment of HeLa cells with DOX and EA, the GSH level decreased to 12.1 µg/10^6 cells, (42%), but similar to the level found in the cells treated with DOX alone. Simultaneously, the GSSG level increased 1.5-fold.

Results from the cytotoxicity test, presented in Figure 1, indicate that co-treatment of the tested cells with EA and resulting changes in GSH content did not significantly affect the IC_{50} values for DOX.

Discussion

As shown by other authors, many drug-resistant cancer cells have higher GSH levels as a result of increased expression of the γ-GCS gene, which can be induced by treatment [4,19,24,27,34,39,55,57]. Tipnis et al. also found that HeLa transfectants overexpressing γ-GCS were 2-fold more resistant to DOX than their parental cells [53]. Therefore, on the basis of literature data, it was initially expected to achieve higher levels of GSH in the resistant cells of both types, which could be explained as a phenomenon contributing to their drug resistance. As mentioned above, in our study such a correlation was reported only for human melanoma cells. In the cells used in this

study, native GSH level could not serve as a biomarker of DOX resistance.

After exposure to DOX, the GSH levels increased in all tested cells except for HeLa cells. The most likely explanation for this observation may be that in HeLa cells GSH is used for antioxidant defense and its content is constantly being lowered. Treatment with DOX definitely generates ROS, so the increase of GSH level observed in the study in most cells after exposure to DOX may be one of the mechanisms of adaptation to the treatment.

The results obtained in the cytotoxicity test seem to refute the hypothesis of a central role of GSH in DOX resistance of the tested cells, as inhibition of γ-GCS activity did not sensitize the tested cells to DOX. These results are consistent with data obtained by other authors. Bellamy et al. reported that depletion of GSH by BSO had no effect on DOX sensitivity in drug-resistant human myeloma cells [5]. Cole et al. also noted that BSO did not enhance DOX cytotoxicity in a multidrug-resistant (MDR) small cell lung cancer cell line [11], and Asakura et al. made the same observations using rat hepatoma cells [2].

In our study, inhibition of the activity of GST also did not have a major effect on the sensitivity of tested cells against DOX, although as we reported it causes significant changes in intracellular GSH levels. On the other hand, accumulation of GSH observed in ME18, ME18/R and KB-V1 cells suggests that some of the GSH may form conjugates. Whether GS-DOX conjugates are formed and whether they are actively transported from the cells via MRP proteins still needs to be verified. It was already observed that many natural product drugs, including DOX, are substrates for MRP1 [31]; however, according to many authors, involvement of GSH in transmembrane transport of DOX is still not evident. It was found previously that human melanoma cells express high levels of GSTM1 and multidrug-resistance protein 1 (MRP1) [38,49]. However, several studies have indicated that activity of GST is probably not a major factor related to sensitivity of human melanoma cells to DOX. Ramachandran, Yuan et al. observed that the increased expression of *GSTπ* and *MDR-1* genes in five human melanoma cell lines was not associated with DOX resistance [46]. They also found that high DOX retention did not have a significant effect on its cytotoxicity against the tested cells. Another study by this research group showed that the observed DOX resistance was probably related to DOX-induced DNA breakage and topoisomerase II activity [45]. Depeille et al. also observed that the sensitivity of human melanoma A375 cells to DOX was not affected by reduction of *GSTP1* expression by antisense RNA [12]. DOX resistance of KB-V1 cells has been attributed mainly to overexpression of the Pgp protein, which is a major factor changing the intracellular drug concentration [50]. However, as shown by Kim et al., Pgp activity was not a critical factor in acquisition of the multidrug resistance phenotype of KB-V1 cells [28]. Involvement of MRP1 in the transport mechanisms underlying drug resistance could possibly explain the phenomenon, but it is still not supported by sufficient evidence.

In our studies, inhibition of GST in HeLa cells does not have a noticeable impact on GSH level: native or after exposure to DOX. This suggests that active transport of GSH-DOX conjugates does not occur. On the other hand, studies of other research groups indicated the potential role of GSH-related transport in drug resistance of HeLa cells. Research by Cao et al. showed that transfection of HeLa cells with *GST-pi* cDNA resulted in decreased sensitivity to DOX compared to cells with lower *GST-pi* expression [10].

Unfortunately, our attempts to use the spectrofluorometric method for direct measurements of GSH released to the culture medium were not successful. Although we achieved LOQ similar to Kamencic et al., the sensitivity and accuracy of the method were insufficient to generate reliable results and still need to be improved. Nevertheless, on the basis of obtained data it could be suggested that in ME18 and HeLa cells treated with DOX, the efflux does actually occur. The possible explanation why it takes place in non-resistant cells rather than in their resistant derivatives could be that GSH efflux may be non-specific and associated with the early stages of apoptosis. In fact, some authors indicated the role of GSH in this process, pointing to GSH transport as a central mechanism mediating redox signaling during cell death progression [16,17,18]. According to the authors, GSH depletion, by its efflux, regulates early apoptosis by modulation of executioner caspase activity in lymphoma cells.

Conclusions

At this point of the studies it is difficult to conclude about mechanisms of DOX resistance in the tested cells. On the basis of the literature data and the obtained results it can be stated that the mechanisms underlying DOX resistance are highly cell-specific. Taking into account the different etiology of melanoma and cervical cancer cells, it was expected that the results obtained in the study would not be similar. Despite observations of different cell-specific effects related to GSH, they do not seem to be essential in terms of DOX resistance.

As a general conclusion, in both melanoma cells and KB-V1 cells, there is a possibility that GSH may be involved in active transport of DOX outside the cells. Whether this mechanism directly involves the MRP pathways still remains unclear. HeLa cells seemed to be devoid of the specific, GSH-related mechanism of DOX resistance. These assumptions, however, need to be fully confirmed in our future research.

Ongoing studies seek to clarify the role of GSH in DOX resistance in the tested cells based on measurements of the activity of other GSH-dependent enzymes, i.e. GPX and GR, as well as expression assays of genes probably involved in DOX resistance mechanisms using RT-qPCR.

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