

INFLUENCE OF CYTOSTATICS ON RELATIVE GENE EXPRESSION IN REDOX STATUS, APOPTOSIS AND MIGRATION COLORECTAL CARCINOMA MODEL SYSTEM

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ABSTRACT. Colorectal cancer is one of the leading causes of mortality worldwide. It is the most common malignancy and there is a need for new approaches in therapies. Surgery, radiation and chemotherapy are the key components of colon cancer treatment. Besides common chemotherapy, alternative therapies are being studied to increase treatment effectiveness and reduce side effects. In this article, colorectal carcinoma cells were treated with chemotherapeutics and relative gene expression was investigated for the genes coding cytoskeleton proteins: *CDH1*, *CTNNB1* and *CDH2*; for redox status genes: *GPX1*, *GPX2*, *GPX3*, *GPX4*, *TXNRD1*, *GSTP1*, *NFE2L2*, *NFKB1*, *HIF1A*; and for apoptosis genes: *CASP3*, *CASP8*, *CASP9*, *FAS*, *BCL-2* and *BAX*. The results of our research showed that some concentrations of chemotherapeutics increased the expression of certain genes. Molecular alterations that lead to colorectal cancer can determine appropriate and effective treatment – chemotherapeutics, as well as the design of direct therapeutic targets.

Keywords: colon cancer, redox status, cell migration, chemotherapy, gene expression.

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent malignancies (VAFAPOUR *et al.*, 2021) and the third most common cancer type worldwide (XI *et al.*, 2021). Mutations in oncogenes, tumour suppressor genes and genes related to DNA repair mechanisms lead to sporadic, inherited, or familial CRC (MÁRMOL *et al.*, 2017). Sporadic carcinoma is one of the main diseases in developed countries and it mostly develops spontaneously because there is no known hereditary predisposition (WEITZ *et al.*, 2005). The earliest phases of carcinoma

initiate in normal mucosa with disruption of cell replication and renewal (PONZ *et al.*, 2001). Therefore, understanding the mechanisms of molecular features such as migration, invasion and apoptosis is crucial (MALKI *et al.*, 2021). Cancer cell migration and invasion into surrounding tissue and vasculature are the initial steps in the progression of tumor infiltration. Invasion is characterized by epithelial-mesenchymal transition (EMT) and these cells can migrate as a single cell or in the form of clusters (YAMAGUCHI *et al.*, 2005). Therefore, local proteolysis of the extracellular matrix, pseudopodial extension, and cell migration are needed (CAREY *et al.*, 2015).

The mechanisms of cell migration of tumor cells are similar to healthy cells during normal physiological processes. In order to migrate, cells must change their shape and motility due to interactions with surrounding tissue structures. Within this, the extracellular matrix provides the substrate and the medium through which they migrate. Firstly, the motile cell becomes polarized and elongates. Then pseudopodia are formed, by elongation of the cells, by which they adhere to the components of the extracellular matrix. The anterior parts or the entire cell body contract, which allows the cells to advance through the medium of the extracellular matrix. Chemokines and growth factors promote the morphological transformation of cells favoring their motility and progression (FRIEDL *et al.*, 2003). Metastases represent the dissemination of malignant cells from the site of the primary tumor to distant organs. These processes allow tumor cells to infiltrate blood and lymphatic vessels as possible routes of dissemination (YAMAGUCHI *et al.*, 2005).

Apoptosis is a process of programmed cell death and plays an important role in a huge number of physiological processes (LAWEN, 2003). When something goes wrong in a cell, they are destroyed by apoptosis. If not, the damaged cell may survive, divide and develop into a cancerous (WANG, 2020). These cells then use mechanisms to protect from the damaging effects of chemotherapy by avoiding apoptosis or reducing intracellular drug accumulation (YANG *et al.*, 2009).

Resistance to cell death is an important aspect that provides advantages during the metastatic process. In numerous physiological processes that use O₂, reactive oxygen species (ROS) are generated as a by-product. When the organism finds itself in stressful situations, it creates an antioxidant defense system. The reduced amount of ROS has a positive effect on the organism, especially on cell signaling where it plays a key role. This increased production of ROS and reduced antioxidant protection can cause cell damage (DNA), oxidation of fatty acids to lipids and amino acids to proteins, and deactivation of certain enzymes and their co-factors (ACHARYA *et al.*, 2010). ROS have been involved in promoting cell survival, for example, anti-apoptotic proteins (*BCL-2*) have been shown to increase intracellular ROS levels but have also been shown to increase metastasis by promoting cell survival (TOCHHAWNG *et al.*, 2013).

Oxidative stress is involved in the formation of malignant tumors through genetic mutations, inhibition of apoptosis and promotion of tumor cell proliferation, differentiation, and migration (ZIŃCZUK *et al.*, 2019). ROS is a target for anticancer drugs because of its function, which can either trigger apoptosis or allow cells to adapt to different environments (KIM *et al.*, 2019). Metastasis could be a tumor strategy for avoiding oxidative damage formed in the primary tumor because ROS could modify the cytoskeleton, enabling cells to acquire the invasive phenotype (SERRANO *et al.*, 2020).

The survival and prognosis of patients are based on the tumor stage at the time of detection (ASLAM *et al.*, 2009) and the most reliable screening tool is colonoscopic screening (NG *et al.*, 2009). Despite these, there is a need for new diagnostic and therapeutic methods to improve the treatment of CRC (BARAN *et al.*, 2018). Also, understanding how metastatic cancer cells are connected to ROS levels could help in the formation of new strategies to prevent cancer progression (TOCHHAWNG *et al.*, 2013).

Many authors were focused on the investigation of commercial chemotherapeutics on relative gene expression (LIANG *et al.*, 2020; REHMAN *et al.*, 2021). Our Google Scholar 2012–2022 analyses of these topics indicate that there are many articles on cancer cell line and chemotherapeutics. When we investigated the combination of words, we have fewer articles (HCT-116 and 5-Fluorouracil – 7.880, HCT-116 and Oxaliplatin – 4.653, HCT-116 and Leucovorin – 1.337, HCT-116 and Irinotecan – 3.598, HCT-116, 5-Fluorouracil and Oxaliplatin – 2.625, HCT-116, 5-Fluorouracil, Oxaliplatin and Leucovorin - 855, HCT-116, 5-Fluorouracil, Oxaliplatin, Leucovorin and Irinotecan - 646). According to our knowledge, the interconnection of the influence of such therapy on redox status, apoptosis and migration potential in the same study is very little investigated (HCT-116, cytostatics, redox status – 4.514, HCT-116, cytostatics, redox status, apoptosis – 4.214, HCT-116, cytostatics, redox status, apoptosis, migratory potential – 2.625). At last, after this analysis, we didn't find a single work that involved all these genes simultaneously and in relation to examining the molecular mechanisms that were the focus of our study. We could understand the results of this study as a contribution to the concept of optimization of the research of cellular mechanisms. Considering that we used the same passage of cells in this research, we believe that our results would contribute to that optimization because literature data on such a methodological approach is scarce.

MATERIALS AND METHODS

Chemicals

For the experiment, were used reagents from different manufacturers with analytical purity. A medium (Dulbecco's modified Eagle – DMEM), Phosphate-Buffered Saline (PBS) and trypsin-EDTA produced by Sigma-Aldrich, Inc., St. Louis, Missouri, USA, were used for cell cultivation. Penicillin/streptomycin and fetal bovine serum (FBS) were obtained from Gipro, USA. Cytostatics were from the oncology department of the University Clinical Center Kragujevac. RNA isolation kit used in the experiment was Isolate II RNA Kit (Bioline, Meridian bioscience, USA). The kit for reverse transcription was the FastGene Scriptase Basic cDNA Kit (NIPPON Genetics EUROPE, Düren, Germany). PCR Kit FastGene 2k IC Green Universal with Fluorescein kit (NIPPON Genetics EUROPE, Düren, Germany) was used to determine relative gene expression. Primers for the PCR reaction were designed and purchased from Invitrogen, Thermo Fisher Scientific, Massachusetts, USA.

Cell cultivation

The adherent colorectal cancer cell line (HCT-116) was obtained from the European Collection of Authenticated Cell Cultures (ECACC). Cells were grown in strictly controlled and sterile conditions, which mimic the physiological conditions of the organism. Cells were maintained in 25cm² culture bottles supplied with 5 mL DMEM supplemented with 10% FBS and 100 µg/mL penicillin/60 µg/mL streptomycin. Physiological conditions for growth were temperature of 37°C, CO₂ concentration of 5%, air humidity of 95% and pH 7.2.

Cells treatment

The chemotherapeutics (5-fluorouracil 50 mg / mL, oxaliplatin 5 mg / mL, irinotecan 40 mg / mL, leucovorin 50 mg / mL) were used in operating concentrations (5-fluorouracil and oxaliplatin 10 and 100 µM, irinotecan and leucovorin 10 and 50 µM). They were previously dissolved in 5 mL DMEM. We choose the treatment concentrations according to the cytotoxicity of the applied chemotherapeutics. the lower concentration was chosen to be not

so toxic to the cells, while the higher one was chosen to be more toxic, according to our previously published results on chemotherapeutics cytotoxicity (DEMETRIADES *et al.*, 2022). After reaching a cell confluence of about 80% we treated the cells in cultivation flasks. The HCT-116 cell line was treated with different concentrations of cytostatics. The time of incubation was 24 h and untreated cells served as a control.

Sample preparation and total RNA isolation

Total RNA was isolated in a laminar hood, using the manufacturer's instructions. After 24 h, RNA was isolated from the cell using a spin colony kit for RNA purification. The kit contains all the necessary reagents to isolate and protect RNA from RNase. The RNA residue was eluted (impurities were removed by washing) and kept in RNase-free water at -20°C.

Reverse transcription and cDNA synthesis

After isolation, the translation of information RNA into complementary DNA (cDNA) was performed using a kit. The reaction was accomplished using Reaction Buffer, MgCl₂, Deoxynucleotide Mix, Oligo-dT Primer, RNase Inhibitor, and Reverse Transcriptase. The total volume of the reaction was 20 µL and the reaction was run in Applied Biosystems Thermocycler in three steps. Aliquots of the cDNA were stored at -20°C and left for further analysis of quantitative polymerase chain reaction (qPCR). In this way, cDNA was obtained, which is much more stable than RNA and can be stored for several months at -20 or -80°C.

Quantitative polymerase chain reaction (qPCR)

qPCR assays of relative gene expression were performed in the Bioengineering Laboratory, Institute of Information Technologies Kragujevac, University of Kragujevac according to laboratory protocols mostly based on MIQE Guidelines (BUSTIN *et al.*, 2009) following GLP standards for PCR detection.

The mRNA levels of 18 candidate genes and of the housekeeping gene were analyzed by RT-qPCR using the FastGene 2xIC Green Universal with Fluorescein kit (Cat. No. LS41, NIPPON Genetics EUROPE, Düren, Germany). This kit is based on using FastGene® IC Green as an intercalating dye, only detecting double-stranded DNA. The RT-qPCR master mix tube contained 100 ng of cDNA, 10 µl 2X FastGene® IC Green, 0.8 µl of forward and 0.8 µl of reverse primer (10 µM) with the addition of PCR-grade water up to 20 µl qPCR reaction. For the cycling reaction, Mic qPCR Cyclor (Biomolecular Systems, Yatala, Australia) was used with additional high-resolution melting analyses. The thermocycling conditions were as follows: 95°C for 2 min; 40 cycles of 95°C for 5 sec and 62°C for 30 sec. Melting analyses were performed at the end of 40 cycles as the proof of reaction product procedure.

Real-time PCR software (micPCR v2.8.13) was used for analyzing the data. Main values (ΔCT) were transferred in Excel and were calculated using the appropriate formula. The $2^{-\Delta\Delta CT}$ formula used to express the differences between observed genes and the housekeeping gene (*GAPDH*) which served as a control was described in the available literature (LIVAK and SCHMITTGEN, 2001; SCHMITTGEN and LIVAK, 2008).

The following genes presented in Table 1 were used:

- for the cytoskeleton: *CDH1*, *CTNNB1* and *CDH2*;
- for redox status: *GPX1*, *GPX2*, *GPX3*, *GPX4*, *TXNRD1*, *GSTP1*, *NFE2L2*, *NFKB1*, *HIF1A*;
- and for apoptosis: *CASP3*, *CASP8*, *CASP9*, *FAS*, *BCL-2* and *BAX*.

Table 1. qPCR primers.

Primers	Forward primer sequence	Reverse primer sequence
CDH1	CAGTCGGTGTATGCCTTCTCG	GAGGGACGCCACATTCTCG
CTNNB1	GGTAGATTTCAATACGTTCCGGG	TGACAGTTCCTCTGATGTCCAAA
CDH2	AGAGCCGGGGACAAGAGAA	ATTGCCAGCATACTGCTTGA
GPX1	GAGGCAAGACCGAAGTAACTAC	CCGAACTGGTTACACGGGAA
GPX2	ACCCATTTTCTACTCAGGACACA	TGCTGGAATACTGTAAGTGTGCT
GPX3	TCTTTTGCATAGAGACCATGACCAG	CTCCCTACTCCAGTAACTCCCGACT
GPX4	ATATGGCAAGAAGGTGATGGTCC	GGGCTTGTCTTAACAAAGCTG
TXNRD1	TGACAATGAGGTTTCTTCGGCT	AATGTCCTGTTGCATACCGTCT
GSTP1	CACAAGGCAGCAAATAGACGAG	TGGGAAATTGTCAGCAGGCTAA
NFE2L2	TCAATCCCACCACGTACAAGG	GCCTCAAAATCCAAGCCCTTT
NFKB1	GACCAGGACTATGACTTGAGCC	AGGGAGTCATATGGTGGAGCT
HIF1A	GCAACCAAGAAAGCAAGCTCAT	TAAAGCTTGCATTCCACCAGC
CASP3	GCTTCAGGGTTTCATCCAGGA	CAATCATCCTCTGCAGCTCCA
CASP8	GATAACGGAGGCTGGGATGC	GACTTCACTTGTGGCCAGAT
CASP9	TGGAAATAAACTGCACCCGGA	TCCTTTCTCTTCACCCAAACA
FAS	TTCAGCAAAGGGGAGGAGTTG	GTGTGTTCCATTCTGTCCCT
BCL-2	ACTTCCCAGGTTTTGTTTCC	CAAGATAAGGCAGGGTGAGGG
BAX	GCACCTGGTTATTATTCTTGGC	GGACTCAAATTCTGTTGCCACC
GAPDH	CTAGGCGCTCACTGTTCTCTC	GCCCAATACGACCAATCCG

Statistics

The data were expressed as mean \pm standard error (SE). All qPCR reactions were performed in triplicates. Statistical significance was determined using the one-way ANOVA test for multiple comparisons, followed by a Dunnett's test when appropriate to compare individual groups with the control. Significance was accepted at P 0.05. The magnitude of the correlation between variables was done using SPSS (Chicago, IL) statistical software package (SPSS for Windows, version 17, 2008).

RESULTS AND DISCUSSION

Relative expression of cytoskeleton genes (CDH1, CTNNB1 and CDH2)

For analysis of the expression of mRNA for the genes which code proteins involved in cytoskeleton structures (*CDH1*, *CTNNB1* and *CDH2*), we used HCT-116 cell line. Results of the effect of used chemotherapeutics on genes that code *E-cadherin*, β -*catenin* and *N-cadherin* were shown in Fig. 1. Effect of the expression on the *CDH1* gene after treatment showed that 5-fluorouracil and oxaliplatin in the concentration of 10 μ M decreased and in the concentration of 100 μ M increased the expression of this gene. On the other hand, irinotecan 10 μ M decreased, while at a concentration of 50 μ M, it decreased the expression slightly. Results of analysis of gene *CTNNB1* showed that 5-fluorouracil increased expression in the concentration of 10 μ M and decreased in the concentration of 100 μ M. Other chemotherapeutics in both concentrations decreased gene expression, besides leucovorin and irinotecan 50 μ M, which increased expression slightly compared to the control. Finally, the results of gene expression of *CDH2* after treatment with 5-fluorouracil in both concentrations significantly increased expression. A comparable result was obtained after treatment with leucovorin in both concentrations. Despite that, after treatment with oxaliplatin in both concentrations, it is noted to decrease expression. Irinotecan in a lower concentration decreased expression, while in a higher increased significantly.

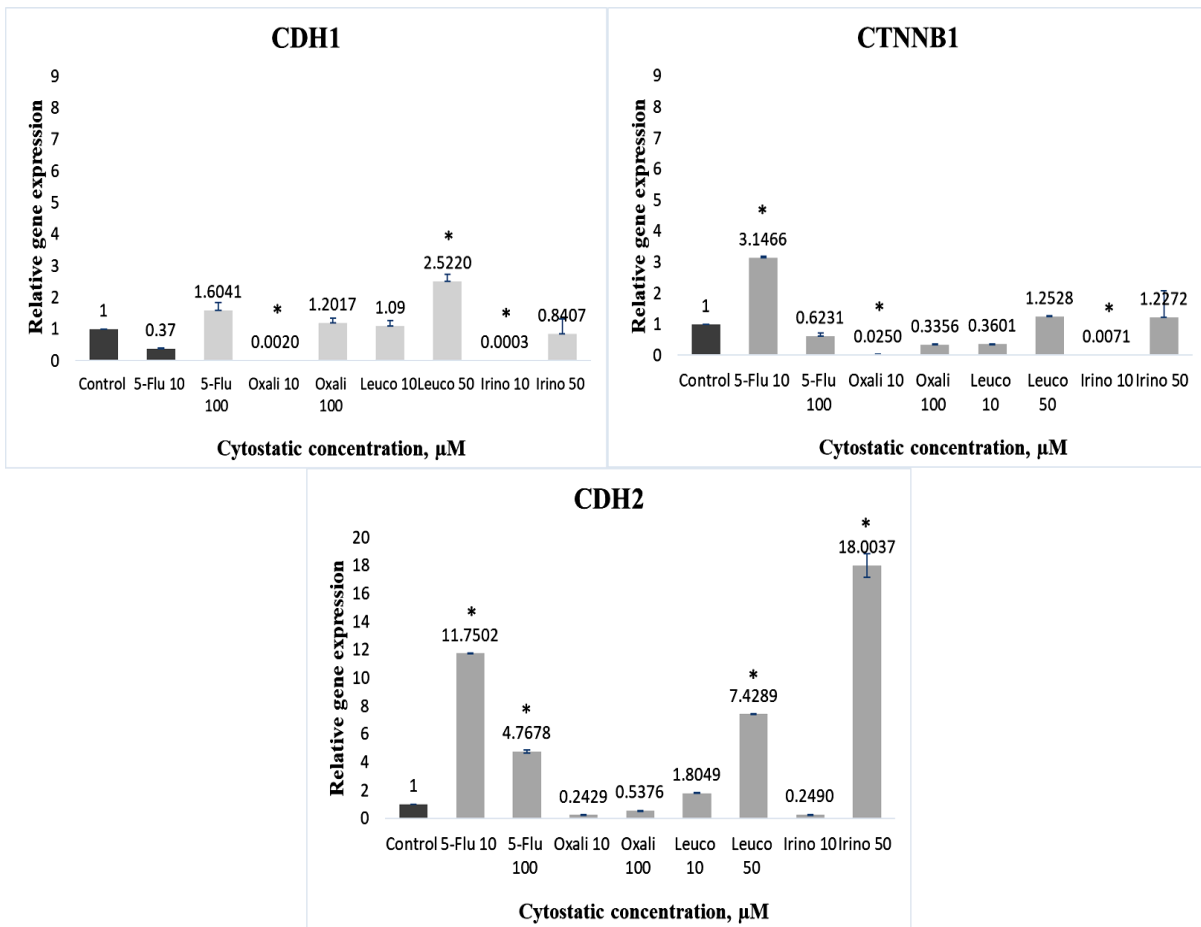


Figure 1. Relative expression of *CDH1*, *CTNNB1* and *CDH2* genes in the treatment of cytostatics on HCT-116 cell line.

The results of the examination of these genes and the expression of their coding proteins are significant in the pathogenesis of tumors, their metastatic potential and the response to the effect of chemotherapeutic agents. As is known, the *CDH1* gene encodes *E-cadherin*, a transmembrane protein that forms adherens junctions on the lateral sides of the epithelial cells, enabling the integrity of the epithelial layer. The results of the research in the available literature pointed out the importance of the adhesive role of this protein in tumor pathogenesis and metastatic process. It has been shown that the reduction of *E-cadherin* adhesive function promotes metastasis. Moreover, mutations of the *CDH1* gene and a consequential reduction in adhesive function contribute to the progression of malignant tumors (PETROVA *et al.*, 2016). This transmembrane protein links to the actin filaments of adjacent cells via the intracellular binding protein β -catenin. The literature data suggest that *N-cadherin* is expressed also in tumor cells in addition to typical localization (intercalated discs, nerve tissue) and that it promotes their transendothelial migration. When a tumor cell adheres to the endothelium, it leads to Src Kinase pathway activation. This activation results in subsequent phosphorylation of β -catenin bound to both *N-cadherin* and *E-cadherin* of endothelial cells. The outcome of these processes is the "opening" of cell-cell junctions and paracellular migration of tumor cells in the subendothelium (RAMIS *et al.*, 2009).

E-cadherin has various functions in different stages of tumor pathogenesis. It is considered that it prevents the initial separation of tumor cells from the primary tumor, and the loss of intercellular junctions allows cells to migrate to distant sites. However, this protein has also been shown to play a role in collective cell migration, both during embryogenesis and

tumor growth, where this protein is an integral component for group-cell chemotaxis *in vivo* (CAI *et al.*, 2014). Moreover, it was shown that although *E-cadherin* inhibits proliferation through contact inhibition (without affecting apoptosis) (Hippo-Yap signaling pathway) (PERRAIS *et al.*, 2007), in some conditions it can stimulate it (MENDONSA *et al.*, 2018).

According to literature data, the HCT-116 cell line, which we also examined, is poorly differentiated, with a high degree of proliferation and a small number of intercellular connections or low expression of *E-cadherin* (DRUZHKOVA *et al.*, 2019). According to the same data, these poorly differentiated cells showed high sensitivity to the effects in chemotherapeutics. In our research, we used these cells to examine the "maturation of the phenotype" and the formation of intercellular connections of poorly differentiated tumors. The results of our study, which showed that 5-fluorouracil in a concentration of 100 μM significantly increased the expression of the gene for *E-cadherin*, could be in agreement with the results of the studies TIGU *et al.* (2020), who showed that 5-fluorouracil in higher concentrations (200 μM) inhibits cell migration almost equally in intact and tumor cells. Our study showed that even at doses lower than 200 μM , inhibition of tumor cell migration could be achieved by the effect of all analyzed cytostatics. This requires further research. Apart from depending on the concentration, as we mentioned previously, the cytostatics achieve different effects depending on the degree of differentiation, which, among other things, is related to the expression of *E-cadherin*.

Cells show resistance to cytostatics due to well-formed connections that prevent the inflow of cytostatics into the tumor. The reason is that *CDH1* acts as a chemoprotective agent by blocking drug distribution through the intercellular junctions (DRUZHKOVA *et al.*, 2019). The results of our study that stand out are the reduced expression of *CTNNB1* and *CDH2* genes in both concentrations under the effect of oxaliplatin. This variation of oxaliplatin could be due to different mechanisms of action, which will be discussed below.

Relative expression of apoptosis genes

The results of the effect of the used cytostatics on caspase gene expression are shown in Fig. 2.

After treatment with a lower concentration of 5-fluorouracil (10 μM), *CASP3* gene expression was stimulated, while a higher concentration (100 μM) reduced *CASP3* expression. In contrast, for the other two caspase genes, these two concentrations had opposite results. The effect of oxaliplatin in both concentrations had a significant effect on the reduction of *CASP3* and *CASP8* gene expression, while *CASP9* expression increased after the application of a higher concentration (100 μM). The Leucovorin 10 μM decreased the expression of *CASP3*, while 50 μM stimulated the expression significantly. *CASP8* gene expression was significantly reduced after all cytostatic concentrations, except for 5-fluorouracil 100 μM . Leucovorin in both concentrations influenced the increase in *CASP9* gene expression, while irinotecan significantly decreased the expression at lower concentrations (10 μM).

FAS and *BCL-2* genes showed decreased expression after treatment with all cytostatics in both concentrations, compared to the control. Despite that, treatment with cytostatics leads to increased *BAX* expression (Fig. 3).

Apoptosis plays a key role in tumor pathogenesis (ASADI *et al.*, 2018) and its mechanisms mainly consist of extrinsic and intrinsic pathways (LOWE and LIN *et al.*, 2000). The extrinsic pathway of apoptosis activation takes place via the "death receptor" *FAS* on the cell membrane. *FAS* is one of the factors of tumor necrosis, it exists as a membrane receptor in all types of mammalian cells. When its ligand is activated, *CASP8* in the death-inducing signaling complex (DISC) is triggered, followed by *CASP3* activation, leading to cell apoptosis (AMETLLER *et al.*, 2010).

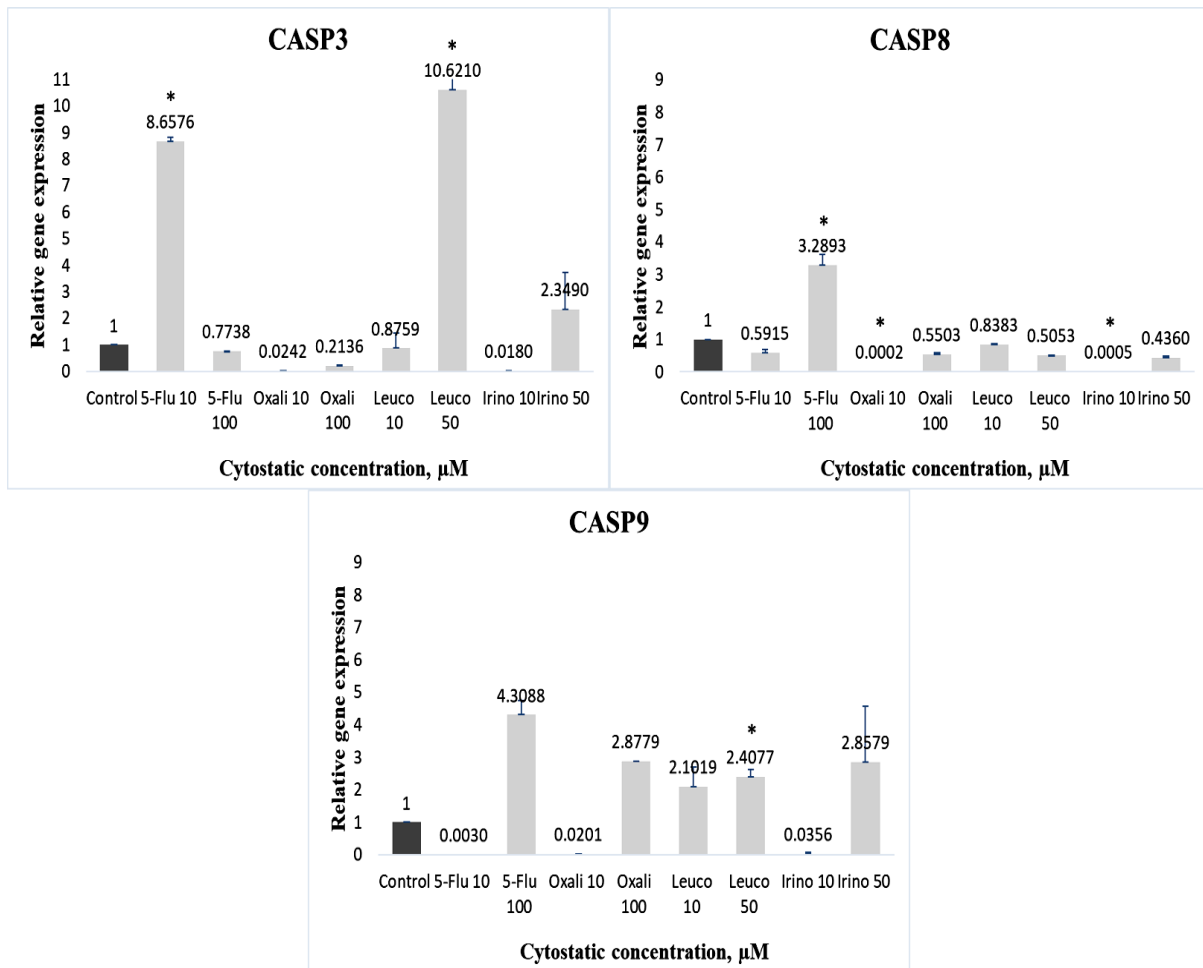


Figure 2. Relative expression of *CASP3*, *CASP8* and *CASP9* genes in the treatment of cytostatics on HCT-116 cell line.

Otherwise, the intrinsic pathway is achieved by signals coming from inside the cell due to various damage to the nucleus or organelles. Such DNA damage activates the tumor suppressor gene p53, which directly regulates the expression of the *BAX* protein. *BAX* is normally found in the cytosol, but under the effect of apoptotic stimuli, it relocates to the outer membrane of mitochondria. Such DNA damage activates the tumor suppressor gene p53, which directly regulates the expression of the protein. *BAX* is normally found in the cytosol, but it relocates to the outer membrane of mitochondria, after the effect of apoptotic stimuli. In this process, the potential of the mitochondrial membrane changes, or otherwise, there is an increased permeability for small molecules such as cytochrome C or ROS. Their movement from the cytosol activates *CASP9* and then *CASP3* as an effector caspase. On the outer membrane of mitochondria, there is also an antiapoptotic protein called *BCL-2* (ARANGO *et al.*, 2004). It is believed that the *BAX/BCL-2* relation can be a factor in tumor prognosis and that a high *BAX/BCL-2* relation favors apoptosis (HOSSEINI *et al.*, 2020). *CASP8* and *CASP9* are initiators, while *CASP3* is an effector of caspases and all three are frequent in tumor pathogenesis (OLSSON and ZHIVOTOVSKY, 2011). What is known is that activated *CASP3* can degrade intracellular structural and functional proteins and cause cell death (JIANG *et al.*, 2020).

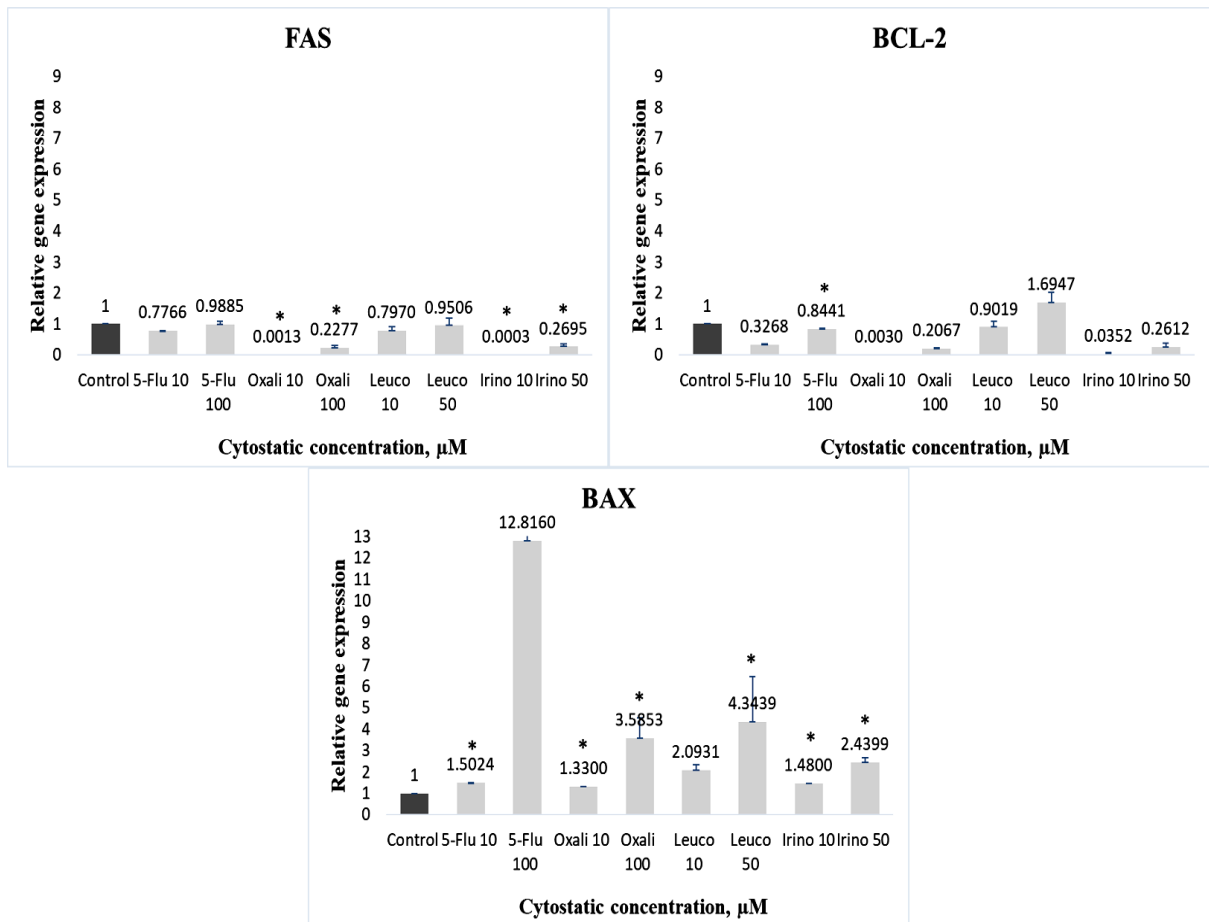


Figure 3. Relative expression of *FAS*, *BCL-2* and *BAX* genes in the treatment of cytostatics on HCT-116 cells.

The results of our study showed that after treatment with cytostatics the expression of the *FAS* gene, as well as *CASP8*, was reduced. This result suggests that the chemotherapeutics used in our study did not induce apoptosis through external activation. Moreover, our results showed increased gene expression for *CASP9* and decreased expression of the *BCL-2* gene. This result suggests that cytostatics achieved their mechanism of action through internal apoptosis. The mechanism of action and the effector effect of *CASP3*, which had a significant increase in low concentrations of 5-fluorouracil, need to be further analyzed in future research.

As we have previously stated, the results of the expression of genes encoding apoptosis factors suggest that the mechanism of action of cytostatics is the internal pathway of apoptosis activation. This result is in accordance with other literature data. Namely, 5-fluorouracil achieves its anticancer effect by inhibiting thymidylate synthetase and incorporating its metabolites into the RNA and DNA of tumor cells. Research has shown that colorectal cancer cells after 5-fluorouracil treatment undergo different degrees of apoptosis depending on the cell line, but that it is a caspase-dependent pathway and that it is achieved through *CASP9* (internal pathway of activation) (MHAI DAT *et al.*, 2014). Then, leucovorin enhances the therapeutic effect of 5-fluorouracil in colon cancer therapy by stabilizing its active metabolite (5-FdUMP) for the enzyme thymidylate synthetase (HEGDE and NAGALLI, 2022). Comparing the effects of cytostatics used in our study with each other, our results suggest that 5-fluorouracil stimulates the apoptosis of freely differentiated cells of our cell line in multiple ways, increasing the expression of *CASP9*, *CASP3* and *BAX* genes. In a future perspective, it would be interesting to examine the synergistic effect of 5-fluorouracil and leucovorin in more detail.

Relative expression of redox balance genes

After the effect of cytostatics, expression of the *GPX1* gene was inhibited compared to the control in all concentrations (Fig. 4). In contrast, expression of the *GPX4* gene was significantly increased. After treatment with 5-fluorouracil (10 and 100 μM) and leucovorin in higher concentrations (50 μM), it leads to increased expression of the *GPX2* gene. Other cytostatics inhibited the expression of this gene. Finally, after treatment with a low concentration of 5-fluorouracil (10 μM), the expression of the *GPX3* gene was reduced, while the application of higher concentrations (100 μM) significantly increased the expression of *GPX3*. Other cytostatics decreased the expression of *GPX3*.

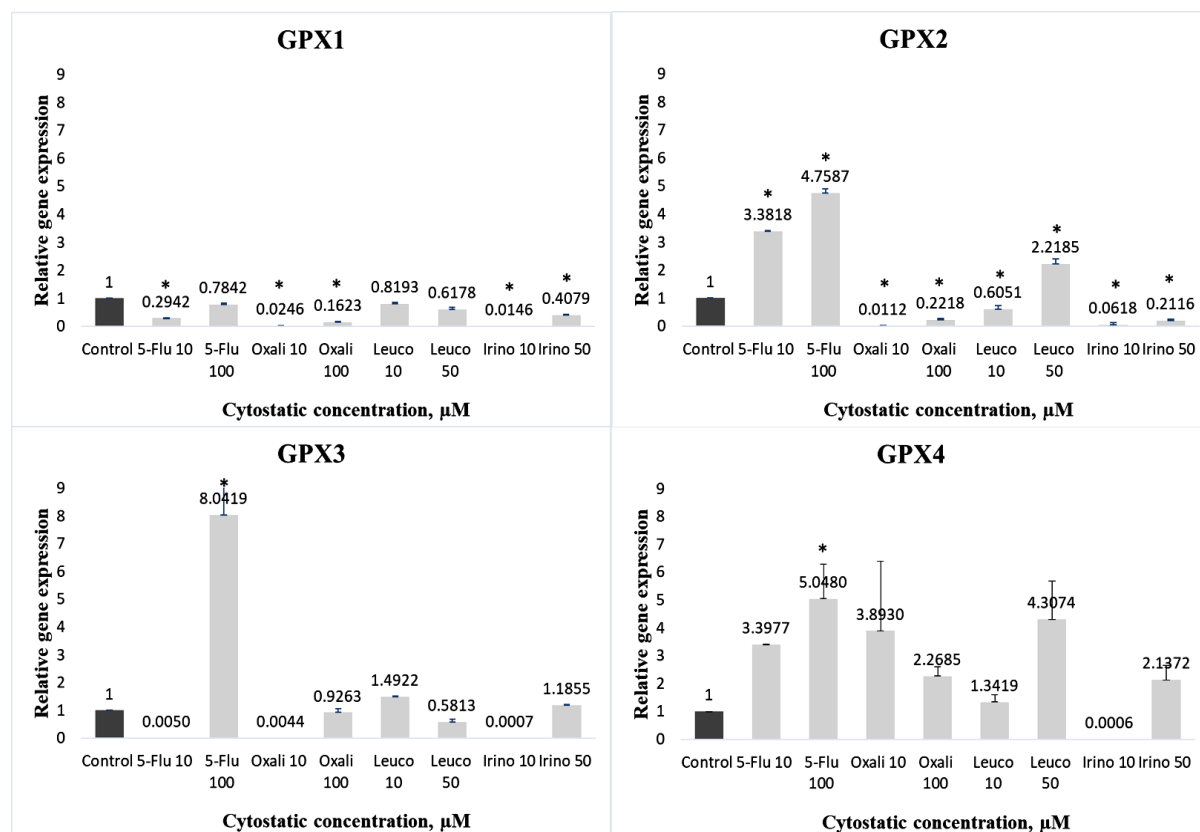


Figure 4. Relative expression of *GPX1*, *GPX2*, *GPX3* and *GPX4* genes in the treatment of cytostatics on HCT-116 cells.

Treatment in concentrations of 5-fluorouracil 100 μM and leucovorin 50 μM slightly stimulated the expression of the *TXNRD1* gene, while treatment with 5-fluorouracil 100 μM minimally increases the expression of the *GSTP1* gene compared to the control (Fig. 5). Expression of *TXNRD1* and *GSTP1* genes were reduced after the effect of cytostatics, and these are genes that are activated when the redox balance is disturbed. In contrast, 5-fluorouracil, leucovorin and irinotecan, in higher concentrations, increase the expression of these genes compared to the control. Also, 5-fluorouracil in a higher concentration increased the expression of the *GSTP1* gene.

The results of the analysis of the transcription factors *NFE2L2* and *NF κ B* showed that treatment with both concentrations of 5-fluorouracil significantly increased their expression. Also, treatment with leucovorin increased *NRF2* gene expression. In contrast, oxaliplatin and irinotecan at lower concentrations decreased gene expression, while at higher concentrations they increased expression (Fig. 6). All concentrations of cytostatics increased expression except oxaliplatin and irinotecan which increased *HIF1A* expression.

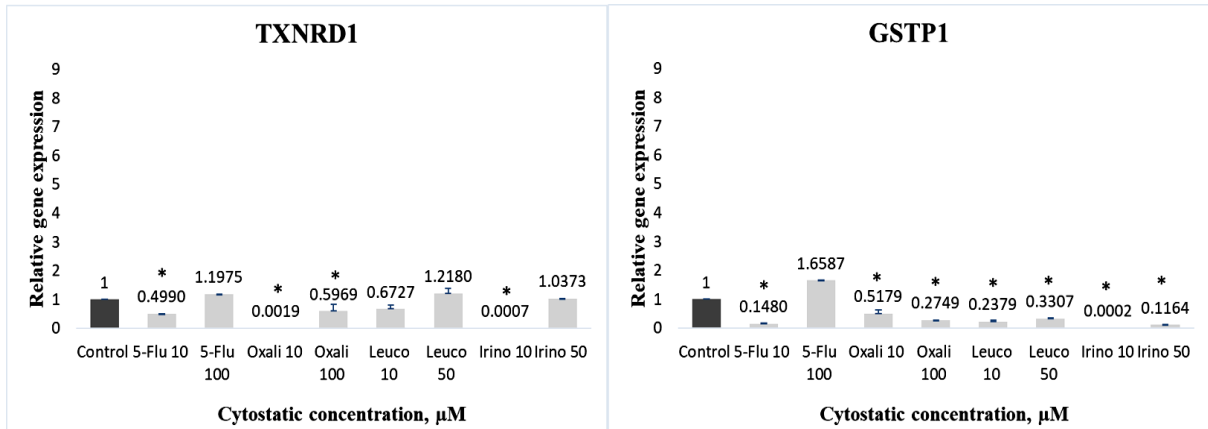


Figure 5. Relative expression of *TXNRD1* and *GSTP1* genes, in the treatment of cytostatics on HCT-116 cells.

Oxygen free radicals or ROS are oxidizing agents that are formed in the process of breathing. Aerobic organisms have consequently developed various protective antioxidant enzymes that reduce oxidative damage, such as the glutathione cycle enzymes that we analyzed in this study. Disruption of this regulation leads to oxidative stress, which is the basis of numerous diseases (WU, 2006). Research has shown that ROS plays an important role in all stages of tumor pathogenesis and tumor metastasis, including the processes of EMT, migration, invasion of cancer cells and angiogenesis, as well as influencing the transcriptional regulation of gene expression involved in these processes. Intracellularly generated large amounts of ROS by growth factors and cytokines (such as *TGF β* and *HGF*) activate cell adhesion proteins, leading to cancer cell migration (WU, 2006).

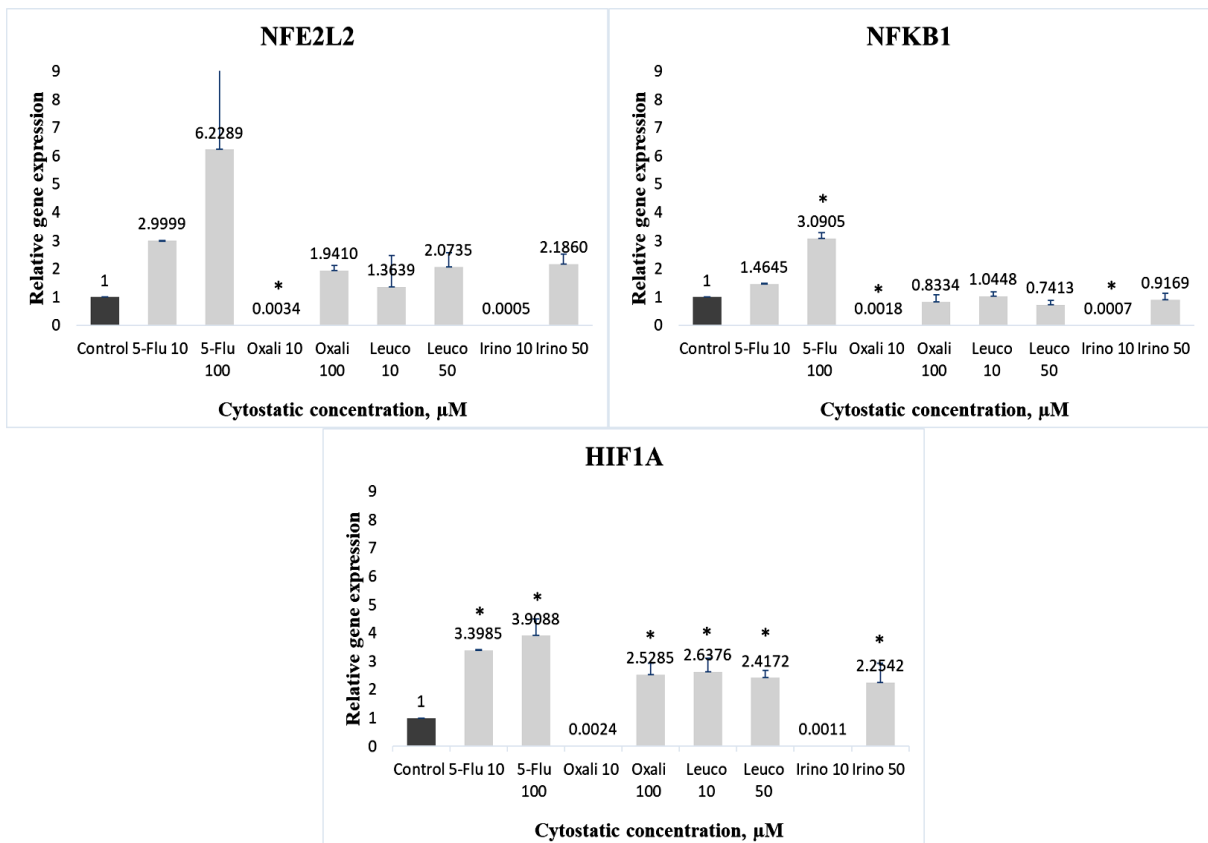


Figure 6. Relative expression of *NFE2L2*, *NFKB1* and *HIF1A* genes, in the treatment of cytostatics on HCT-116 cells.

The genes analyzed in our research, *GPX1*, *GPX2*, *GPX3*, *GPX4*, *TXNRD1* and *GSTP1* encode proteins of the same name that produce the glutathione cycle. These enzymes of the glutathione cycle perform their effect through an NADPH-dependent redox regulation system, protecting the cell from oxidative stress and ROS (ESPINOSA-DIEZ *et al.*, 2015). The *GPx* genes are the genes that encode the proteins for the corresponding members of glutathione families of peroxidases, which catalyze the reduction of hydrogen peroxide by glutathione and protect cells from oxidative damage (BRIGELIUS-FLOHÉ and MAIORINO, 2013). In humans, eight of them are expressed and most of them are selenoproteins (*GPX1*, *GPX2*, *GPX3*, *GPX4*, *GPX6*). They perform their antioxidant effect in different parts of cells: *GPX1* is found in the cytosol, nucleus and mitochondria; *GPX2* accumulates in the cytosol and nucleus; *GPX3* is also found in the cytosol, while *GPx4* is present in the nucleus, cytosol, and mitochondria and binds to the membrane (LIANG *et al.*, 2009). However, recent research points to the bifunctional nature of these, in principle, protective enzymes in tumor pathogenesis, which we will briefly discuss below.

The *GPX1* gene encodes an enzyme involved in antioxidant defense and is responsible for protecting the cell from the harmful effects of free radicals. In patients with colon cancer, it was determined increased level of *GPX1* gene expression, compared to normal tissue (NALKIRAN *et al.*, 2015). Increased *GPX1* expression is associated with higher levels of oxidative stress, present in tumor cells which could be a contributing factor in tumor pathogenesis (ZHAO *et al.*, 2022). Studies have shown the protective role of higher expression of the *GPX1* gene in cell survival (VIBET *et al.*, 2008). Nevertheless, the normal effect of *GPX1* to lower cytotoxicity which was induced by oxidative stress in normal cells becomes counterproductive in tumor cells and supports their survival and growth (BRIGELIUS-FLOHÉ and KIPP, 2009).

Nevertheless, it has been shown experimentally that *GPX2* has a dual role in carcinogenesis. On the one hand, it protects the cell from carcinogenesis, while on the other hand, it promotes the growth of tumor cells (EMMINK *et al.*, 2014). There is a possibility that *GPX2*, like other selenoproteins and most likely selenium itself, operates differently depending on the tumor type and the presence or absence of inflammation. Also, it was shown that with reduced expression of *GPX2*, compensatory increases expression of *GPX1* (MÜLLER *et al.*, 2013). By comparing such literature data with the results obtained in our study, we note that the concentration of *GPX1* and *GPX2* decreased after the effects of all cytostatics, except in the case of 5-fluorouracil and leucovorin at higher concentrations. This result suggests that the effect of applied cytostatics on poorly differentiated cells in our study reduced the protective properties of the cells themselves and that a good response to therapy was thereby achieved. In the case of 5-fluorouracil, the obtained result would suggest that *GPX2* is up-regulated, most likely to compensate for the loss of *GPX1*, but this is only an assumption that would require further research.

GPX3 has a dichotomous role in different types of tumors, acting as both a tumor suppressor and a pro-survival protein (CHANG *et al.*, 2020). The important role of this gene in CRC patients was described by PELOSOF *et al.* (2017), where reduced gene expression was associated with an increase in ROS. The cellular antioxidant enzyme glutathione peroxidase 4 (*GPX4*) is crucial for the regulation of oxidative stress and ferroptotic cell death and could have an oncogenic role in the development of multiple cancers (SHEN *et al.*, 2021). Increased *GPX4* expression is associated with higher levels of glutathione, which can help neutralize oxidative damage in the cell and protect against the development and progression of cancer (LIU *et al.*, 2021). Upregulation or activation of the intracellular level of *GPX4* can cause cell resistance to ferroptosis (a new neoapoptotic mode of cell death), suppress the therapeutic tumor effects of drugs, and finally result in resistance (YANG *et al.*, 2021). Literature data indicate that *GPX4* plays a role in tumor resistance to chemotherapy, which suggests that the

inhibitory effects of *GPX4* together with chemotherapy or targeted therapy would allow the full effect (ZHANG *et al.*, 2022). Our results showed decreased expression of *GPX3* after all cytostatics, which compared to previous literature data would suggest that cytostatics applied to poorly differentiated cells of our cell line affected the reduction of the defense ability of tumor cells and achieved their effect. Our results also showed up-regulation of *GPX4* after the application of all cytostatics, which is in accordance with literature data and suggests that *GPX4* inhibitors should be used along with chemotherapeutics.

Thioredoxin reductases are proteins encoded by three individual genes, which show different subcellular localization and tissue expression. *TXNRD1* is involved in regulating the expression of genes associated with apoptosis (programmed cell death), oxidative stress, DNA damage, and cell cycle control. Mutations in this gene can lead to the unregulated growth of cells, which can then lead to the development of tumors. In tumors, inhibition of this gene has been suggested to increase the sensitivity of cancer cells and stimulate apoptosis. It has been proposed that *TXNRD1* cooperates with the *GPX4* to protect cells from the lethal accumulation of lipid peroxides, but this requests further research. Increased expression of *TXNRD1* is related to advanced tumor progression and metastasis and chemotherapeutic resistance (GAO *et al.*, 2020). This indicates that the cells are under increased oxidative stress, which can be caused by a variety of factors including genetic mutations, environmental stressors, or excessive energy production. This increase in oxidative stress can lead to increased cell proliferation and survival, which can then contribute to tumor growth and progression (INGOLD and CONRAD, 2018).

Glutathione S-transferases (*GSTs*) form a family of proteins with seven members of similar structure and function encoded by genes of the same name. They represent important antioxidant enzymes that regulate signaling pathways induced by oxidative stress. Numerous studies have shown that their role is complex and multiple and that it participates in cell survival, proliferation and resistance to cytostatics. (SINGH *et al.*, 2021). Research has shown that enzymes involved in redox homeostasis maintenance can detoxify chemotherapeutic drugs (SHEN *et al.*, 1997). In other studies, a positive correlation was found between overexpression of *GSTP1* and chemoresistance in tumors (HUANG *et al.*, 1997).

Comparing the literature data mentioned previously with the results of our studies, we can notice that all cytostatics used in our research lower the expression of both *GSTP1* and *TXNRD1* in poorly differentiated cells of our cell line. The only exception is 5-fluorouracil, which in higher doses increases the expression of the *GSTP1* gene, which suggests that poorly differentiated tumor cells develop resistance to this cytostatic through enzymes that participate in the maintenance of redox homeostasis. The obtained results point to the dose-dependent development of resistance, which is the subject of numerous studies aimed at improving therapy and overcoming this main problem in the application of 5-fluorouracil.

ROS-induced expression of *NFE2L2*, *HIF1A* and *NFKB1* are processes that favor the survival of both healthy and cancer cells which we will explain below (REUTER *et al.*, 2010). The *NFKB1* gene is a transcription factor that has been activated in many malignancies including breast, prostate, pancreas, and colon. Activation of these genes regulates the expression of genes involved in inflammation, cell cycle control, apoptosis, and cell growth. Intercellular levels of ROS have been shown to have an impact on *NFKB1* regulation, but the exact molecular mechanism involved in this regulation is not clear (ACHARYA *et al.*, 2010). It is reported that the increased expression of *NFKB1* in CRC plays an important role in the pathogenesis of colon cancer in humans (ABDULLAH *et al.*, 2013). In our research, cytostatics affected the reduction of the *NFKB1* gene, except for 5-fluorouracil which increased the expression of this gene. High expression of *NFKB1* is specific in cancers, and there is an idea that tumor cells may rely on this pathway to escape from apoptosis (XIA *et al.*, 2014), which would have an impact on chemotherapy resistance.

NFE2L2 is a member of transcription factors and is essential for the regulation of genes that encode stress-responsive or cytoprotective enzymes and related proteins (*NQO1*, *SODs*, *GSTs*, *GPx* and others). This gene plays an essential role in cellular homeostasis maintenance and represents a critical target for oxidative stress prevention (ACHARYA *et al.*, 2010). Our results showed that all concentrations of chemotherapeutics increased gene expression except oxaliplatin and irinotecan which decreased expression.

Increased expression of *HIF-1A* is shown in many tumors, especially in carcinomas (BOS *et al.*, 2001). Namely, as cancers have the potential of initiating a new process of angiogenesis in hypoxic conditions (PANG and POON, 2006), it is not surprising that the investigated cytostatics show the effect of increased induction of *HIF-1A* expression.

CONCLUSION

Tested chemotherapeutics increased the redox stress of certain genes in our experiment. Accordingly, it is known that increased redox stress in cells introduces cells to apoptosis, which we also noticed in our results. All our results agree with the stated mechanisms of action of cytostatics. Also, increased apoptosis and redox stress affect the reduction of the metastatic potential of cells by suppressing a certain gene important for the expression of proteins important in the construction of the cytoskeleton and intercellular communication. This is in accordance with our results as well, especially after 5-fluorouracil treatment. According to our knowledge, this is one of the few studies that methodologically rely on examining the impact of cytostatics. It was important to demonstrate the importance of examining multiple parameters of various cellular molecular mechanisms from the same passage of cells in order to obtain a current cross-section of the state of a very dynamic natural system, such as a cancer cell. Certainly, in the following studies, we will simultaneously deal with the examination of relative gene and protein expression to expand this methodological concept.

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