# 2,4-dinitrophenol enhances cisplatin and etoposide cytotoxic activity on prostate cancer cell line

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## ▌Abstract

**Introduction.** Including additional compounds that disturb the energy metabolism of cancer cells in advanced cancer therapy regimens may be an approach to overcome the problem of drug resistance and the therapeutic effectiveness of classic chemotherapeutics. One of the compounds that decouple oxidative phosphorylation, and thus alter the activity of energy-producing pathways, is 2,4-DNP (2,4- dinitrophenol).
**Objective.** The aim of the study was to assess the ability of the 2,4-DNP to sensitize prostate cancer cells to the action of cisplatin and etoposide, or to intensify their action.
**Materials and method.** The research was carried out on three prostate cancer cell lines (LNCaP, PC-3, DU-145. To assess the effect of cisplatin or etoposide with 2,4-DNP on prostate cancer cells, MTT assay, analysis of the cell cycle and apoptosis detection was performed. Oxidative stress was investigated by CellRox fluorescence staining and expression of genes related to antioxidant defence. In addition, analysis was conducted of the expression of genes related to cell cycle inhibition, transporters associated with multi-drug resistance and DNA repair.
**Results.** The study showed that the simultaneous incubation of 2,4-DNP with cisplatin or etoposide enhances the cytotoxic effect of the chemotherapeutic agent only in LNCaP cells (oxidative phenotype).
**Conclusions.** The enhanced cytotoxic effect of chemotherapeutics by 2,4-DNP may be the result of disturbed redox balance, reduced ability of cells to repair DNA, and the oxidative metabolic phenotype of prostate cancer cells.

## ▌Key words

prostate cancer, cisplatin, uncoupler, etoposide.

## INTRODUCTION

Despite numerous therapy approaches and considerable improvements in science, cancer remains one of the top causes of death globally, with prostate cancer ranking second among men [1]. Only patients with localized prostate cancer may be completely treated due to the development of treatment resistance throughout progression of the the disease. The evidence collected about neoplastic diseases throughout the decades enables the quest for novel therapeutic alternatives for patients with advanced-stage diseases. On the one hand, such a strategy should sensitize the tumour to the administered therapy while also allowing the use of a lower dose of an anticancer drug to minimize its negative effects. One of these approaches might be to target cancer energy metabolism in conjunction with traditional chemotherapeutic drugs. The usefulness of this treatment strategy may be due to the fact that neoplastic cells have a metabolism distinctive from than

normal cells [2, 3]. Cancer cell metabolism is influenced by a variety of variables, including the microenvironment and the growing number of mutations collected during the growth of the tumour [4, 5]. Furthermore, cancer cells change their microenvironment in order to adapt to adverse development conditions [6].

2,4-dinitrophenol (2,4-DNP) is one example of a compound that impairs cellular metabolism. It is well-known for its ability to decouple oxidative phosphorylation, resulting in decreased ATP generation in cells. Changes in the potential of the mitochondrial membrane allow energy to be distributed in the form of heat, skipping the ATP production step [7–9]. Furthermore, it affects the redox equilibrium in cancer cells [10]. In this work, 2,4-DNP was utilized as a mitochondrial function disruptor.

Etoposide and cisplatin chemotherapy (EP) is a combination chemotherapy treatment used to treat different types of cancer. Typically, these two drugs are given one after the other on the same day. The combination of these drugs is highly effective and well tolerated in advanced thymoma, small cell lung cancer, testicular cancer, germ cell tumours [11–13]. Cisplatin is classified as an alkylating agent. Its

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mechanism is mostly dependent on the creation of DNA adducts, which inhibits DNA replication and transcription, hence inhibiting protein synthesis. Furthermore, it causes mitochondrial injury, resulting in oxidative stress in cancer cells, which increases DNA damage and, ultimately, activation of programmed cell death pathways [14].

In turn, etoposide is a semisynthetic podophyllotoxin derived from the root of *Podophyllum peltatum* (May apple or mandrake), which blocks cancer cell division in the late S-G2 phase of the cell cycle via inhibition of Topoisomerase II. At the same time, etoposide, by forming a ternary complex with topoisomerase II and DNA, induces DNA damage through its strand breakage [15]. In addition, there are more and more reports about its ability to induce mitochondrial damage and increase reactive oxygen species (ROS) production [16].

The resistance of cancers to chemotherapeutics is due to the high oxidative defence potential of cancer cells and the large amount of energy they produce, which is necessary for complex DNA repair processes, including DNA ligation [17, 18]. In addition, the presence of multidrug resistance transporters (MDR) is of great importance in the resistance of cancer cells to treatment. The above-mentioned proteins are responsible for e.t. pumping out the chemotherapeutic agent from the inside of the cell and redistributing intracellular accumulation of the drug away from target organelles. These transporters known also as ATP-dependent efflux pumps, which are responsible for the resistance to cisplatin and etoposide [19]. Therefore, it seems reasonable to use a compound that reduces energy (ATP) production (2,4-DNP) in cancer cells in order to prevent the activity of MDR proteins.

Three prostate cancer cell lines with possibly diverse metabolic phenotypes were used in the study, the aim of which was to sensitize the prostate cancer cells to the action of the cisplatin and etoposide, or to intensify their action through the use of the 2,4-DNP

## MATERIALS AND METHOD

**Cell Culture and Treatment.** Prostate cancer cell lines were used in this study due to previous research on their metabolic phenotype [20, 21]. The study was conducted on three prostate cancer cell lines: PC-3, DU-145 and LNCaP (ATCC, Manassas, VA, USA). PC-3 cells were cultured in Kaighn's Modification of Ham's F-12 Medium (F12-K) (Corning, New York, NY, USA), DU-145 cells in Eagle's Minimum Essential Medium (EMEM) and LNCaP cells in RPMI-1640 Medium (Corning, New York, NY, USA). The media were supplemented with 10% FBS (foetal bovine serum, Life Technologies, Carlsbad, CA, USA), penicillin (100 units) and streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Cell lines were maintained within a humidified 5% CO2 atmosphere at 37 °C.

Prostate cancer cells were treated with 100 μM 2,4-DNP and following chemotherapeutics: cisplatin and etoposide (1- 40 μM) or combined (2,4-DNP + CIS/ETO) for 48 h. Used concentration of 2,4-DNP was evaluated on the basis of the previously published studies, clinically achievable plasma concentrations and the observed cytotoxicity for the investigated cells [21].

**Cytotoxicity Analysis.** To evaluate the cytotoxic effect of cisplatin and etoposide alone or in combination with 2,4-DNP on tested prostate cancer cell line, an MTT Cell Proliferation Assay Kit (Invi-trogen, Waltham, MA, USA) was used. The cells were seeded in 96-well plates in a concentration $1.5 \times 10^5$ cells/mL and cultured until 70–80% confluence was obtained. MTT colorimetric assay is based on the ability of viable cells to transform orange tetrazolium salts (3-[4,5-dimethylthiazol-2-yl]-2,5-) diphenyltetrazolium bromide) to a purple formazan product. Following 48 h incubation with the tested compounds, the MTT solution (0.5 mg/mL in phosphate-buffered saline) was added into the wells. Incubation was continued for 3 h at 37 °C. The medium with MTT was then removed, and the colour product obtained was dissolved in 200 μL of DMSO. The absorbance of the resulting solution was measured spectrophotometrically using a PowerWave™ microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) at 570 nm. Each experiment was carried out three times and measurements were conducted in triplicates.

To additionally assess the cytotoxicity of tested compounds, the morphology of the cells was investigated using a phase-contrast microscope – Nikon Eclipse Ti, and NIS-Elements Imaging Software (Nikon, Tokyo, Japan).

**Cell Cycle Analysis.** Cell cycle analysis was performed with the NucleoCounter NC-3000 (ChemoMetec, Allerod, Denmark) in accordance to 2-Step Cell Cycle Assay protocol recommended by the manufacturer. The experiment was evaluated after 48 h incubation of prostate cancer cells with tested compounds. Analysis was performed in line with previously described methodology [22, 23].

**Apoptosis detection.** Detection of apoptosis in tested prostate cancer cells was conducted with the Nucle-oCounter NC-3000 (ChemoMetec, Allerod, Denmark) in accordance with the Annexin V Apoptosis Assay. Analysis was evaluated after 48 h incubation of prostate cancer cells with tested compounds, and performed in line with previously described methodology [24, 25].

**Oxidative stress detection.** Detection of the ROS in cells was conducted using the CellROX Green Reagent (Invi-trogen, Waltham, MA, USA) – a fluorogenic probe. The experiment was performed after 12 h incubation of prostate cancer cells with tested compounds. Analysis was performed in line with previously described methodology [22, 26].

*Quantitative Real- Time PCR analysis (qRT-PCR).* Expression levels of genes were determined by quantitative real-time PCR (qRT-PCR). Cells were seeded into 25 cm3 flasks at a concentration of $1.5 \times 10^5$ cells/mL, and test compounds were added when 70–80% confluence was reached. After 24 h incubation, cells were harvested using trypsin, and lysis via 1 ml of TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA). Next, RNA was isolated according to the Chomczynski and Sacchi method [27]. Obtained RNA were reverse transcribed using NG dART RT-PCR reagents (EURx, Gdańsk, Poland) according to the manufactureer's instructions. The qPCR reaction was performed in triplicate according to the manufacturer's instructions using Fast SG /ROX qPCR Master Mix reagents (2x) (EURx, Gdansk, Poland) in a 7500 Fast Real-Time PCR system (ThermoFisher,

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**Table 1.** qPCR primers used in the experiment

| Gene | Protein name | Forward Sequence (5′ 3′) | Reverse Sequence (5′ 3′) |
|---|---|---|---|
| CDKN1A | Cyclin dependent kinase inhibitor 1A /CDKN1A (p21) | CCTCATCCCGTGTTCTCCTTT | GTACCACCCAGCGGACAAGT |
| CAT | Catalase | AGCTTAGCGTTCATCCGTGT | TCCAATCATCCGTCAAAACA |
| GPX1 | Glutathione peroxidase 1 | TTGACATCGAGCCTGACATC | ACTGGGATCAACAGGACCAG |
| NFE2L2 | NFE2 like bZIP transcription factor 2 | GCGACGGAAAGAGTATGAGC | GTTGGCAGATCCACTGGTTT |
| SOD2 | superoxide dismutase 2 | CTTCAGGGTGGTATGGCTGT | TGGCCAGACCTTAATGTTCC |
| ABCC1 | Multidrug resistance-associated protein 1 (MRP1) | AGGTGGACCTGTTTCGTGAC | ACCCTGTGATCCACCAGAAG |
| ABCC4 | Multidrug resistance-associated protein 4 (MRP4) | GGTTCCCCTTGGAATCATTT | ATCCTGGTGTGCATCAAACA |
| BRCA1 | Breast cancer type 1 susceptibility protein | TTGCGGAGGAAAATGGGTAGTTA | TGTGCCAAGGGTGAATGATGAAG |
| ATM | ataxia telangiectasia mutated protein kinase | GCCGCGGTTGATACTACTTTG | GCAGCAGGGTGACAATAAACA |
| ERCC3 | ERCC excision repair 3, TFIIH core complex helicase subunit (XPB) | CTCGGAGTTTTGTGGGGGAC | CACTGGCGTCTACGTTCTCA |
| MLH1 | MutL homolog 1 | GCACCGGGATCAGGAAAGAA | GCCTCACCTCGAAAGCCATA |
| MSH2 | MutS homolog 2 | CAGGAGGTGAGGAGGTTTCG | CCGTGCGCCGTATAGAAGTC |
| OGG1 | 8-oxoguanine DNA glycosylase | CCTGTGGGGACCTTATGCTG | TGTGAATCCCCTCTCCCGAT |
| RNA18S | 18S ribosomal N5 | GAAACTGCGAATGGCTCATTAAA | CACAGTTATCCAAGTGGGAGAGG |
| BACT | Beta-actin | AGAGCTACGAGCTGCCTGAC | AGCACTGTGTTGGCGTACAG |

USA). *18SRNA* and *BACT* served as reference genes. The relative expression of the studied genes was determined by qRT-PCR and ΔΔCt. Statistical analysis was performed using RQ values (relative quantification, RQ = $2^{-\Delta\Delta Ct}$). The primer sequences are summarized in Table 1.

**Statistical Analysis.** The results were presented as mean ± standard deviation (SD) and analyzed using STATISTICA 13 software (StatSoft, Kraków, Poland). The values were compared using one-way analysis of variance (ANOVA) and *post hoc* multiple comparisons with Tukey's honest significant difference test (Tukey's HSD test). The results were considered statistically significant if the p-value was less than 0.05.

## RESULTS

**Cytotoxic activity.** Initial screening studies were performed on three prostate cancer lines in order to determine their sensitivity to CIS and ETO, as well as to select the appropriate concentration to examine the combined effect with 2,4-DNP. Both chemotherapeutics were used in a wide range of concentrations (1–40 μM) based on literature data [28, 29]. MTT assay indicated that both CIS and ETO cause dose-dependent reduction of the cell viability in all tested prostate cancer cell lines (Fig. 1A and 1B). The LNCaP cells showed greater sensitivity to the cisplatin compared to the other two cell lines. The $IC_{50}$ values for cisplatin were only attained in the instance of the LNCaP cell line ($IC_{50}$ CIS: 31.52 μM) (Fig. 1A). Thus, for the LNCaP cells, a concentration of 20 μM cisplatin was chosen for future investigations, but for the PC-3 and DU-145 cells, the maximum concentration (40 μM) of the tested chemical was chosen. The greatest concentration of cisplatin reduced the viability of PC-3 and DU-145 cells to roughly 60%. No $IC_{50}$ values were obtained for etoposide on the prostate cancer cell lines at the tested range of concentrations (Fig. 1B). As a result, the concentration of 40 μM etoposide was chosen for future research.

In this study, 2,4-DNP was utilized as an uncoupler. The concentration of 2,4-DNP at 100 μM was within the $IC_{20-40}$ range for the studied cell lines; hence, this concentration
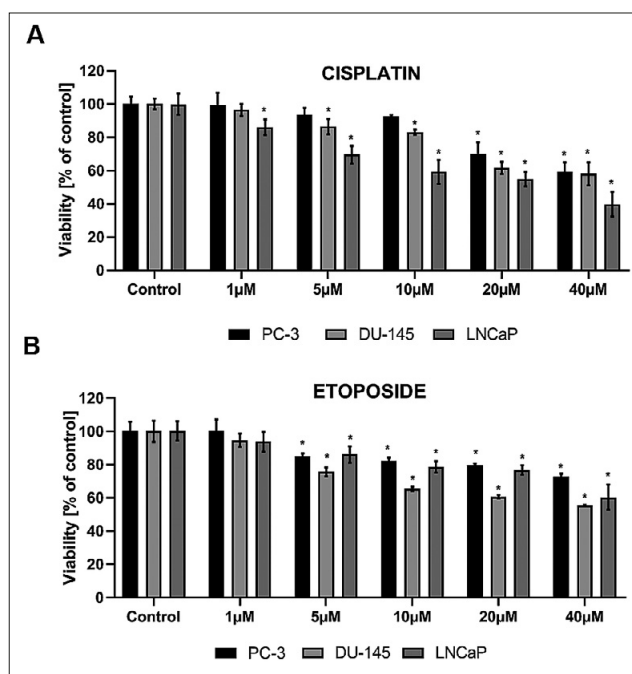


**Figure 1.** Prostate cancer cell lines (PC-3, DU-145, LNCaP) viability (% of control) based on MTT assay. Cells were treated with a wide range of (A) cisplatin (1–40 μM) and (B) etoposide (1–40 μM) concentrations for 48 h. The values obtained from three independent experiments were presented as mean ± SD.
* p < 0.05 vs. control

was chosen for future research. The chosen concentration was lower than the concentration range determined in the biological fluids of patients demonstrating symptoms of poisoning [8–10,30]. To evaluate the effects of the decoupling of oxidative phosphorylation to the cytotoxicity of cisplatin and etoposide, the prostate cancer cells were incubated simultaneously with both the uncoupler (2,4-DNP) and mentioned compounds for 48 h (Fig. 2). Only in the case of the LNCaP cell line, simultaneous incubation of cells with both the 2,4-DNP and cisplatin or etoposide resulted in a statistically significant decrease in cell viability (below 30%), compared to cell viability incubated with each compound alone (Fig.e 2C).
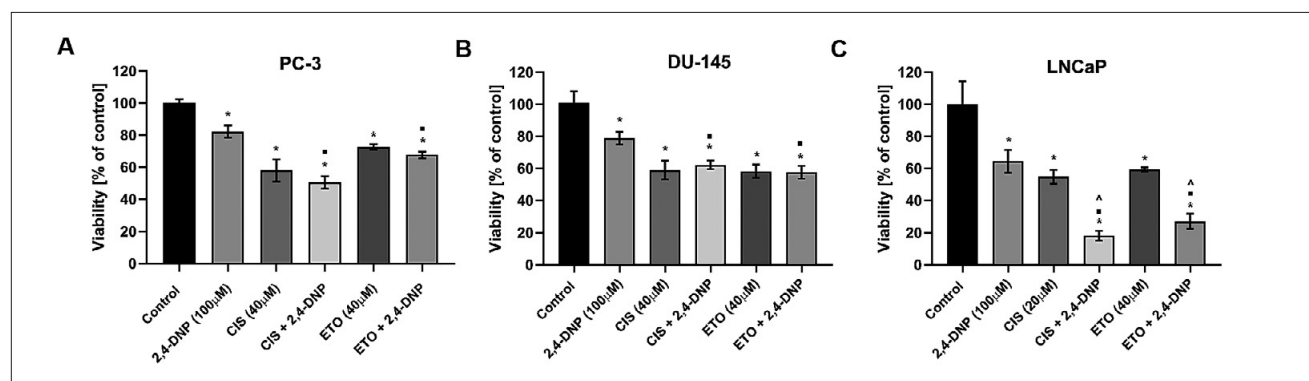
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**Figure 2.** Prostate cancer cell lines (A) PC-3, (B) DU-145, (C) LNCaP viability (% of control) based on MTT assay. The cells were treated with 100μM 2,4-DNP and CIS (cisplatin), ETO (etoposide) or combined (2,4-DNP + CIS/ETO) for 48 h. The values obtained from three independent experiments were presented as mean ± SD. * $p < 0.05$ vs. control. ▪ $p < 0.05$ vs. 2,4-DNP; ^ $p < 0.05$ vs. CIS, ETO

As a result, and also for the confirmation of the observed effect, only the morphology of LNCaP cells was examined using a Nikon Eclipse Ti phase-contrast microscope (Fig. 3), which confirmed the results obtained from the MTT assay (Fig. 3). The morphology of cells incubated with tested compounds alone did not differ from control cells. Only the number of cells in the field of view visible decreased in these cases. In contrast, the simultaneous incubation of cells with both 2,4-DNP and cisplatin or etoposide resulted in many cell morphological changes. Numerous cells observed in the field of view were shrunken, detached from the plate and dead, as well as apoptotic cells also being observed. In addition, the number of cells was clearly reduced, compared to control cells.

To investigate the mechanism of action of the tested compounds, as well as to detect their possible enhancement effect, the rest of the tests were carried out only on the LNCaP line. The cell cycle study of LNCaP cells showed that using 2,4-DNP alone significantly reduced the population of cells in the G1 phase, while increasing the peak of the subG1 phase (equivalent to apoptotic cells)(Figure 4A). On the studied cells, cisplatin/etoposide showed comparable histogram patterns – both drugs effectively suppressed cell proliferation, as evidenced by an increased proportion of cells in both phases the S (about 10-times) and G2/M (over 3-times), compared to control cells. At the same time, a significant increase in the subG1 peak was noted. The simultaneous incubation of LNCaP cells with 2,4-DNP and tested drugs resulted in a significant increase in the number of cells in the subG1 phase of the cell cycle (to about 80%), while the percentage of cells in the G1 phase decreases dramatically when compared to cells incubated with each compound separately, as well as the control. Other phases, such as S and G2/M, were close to zero in both cases. Consequently, the above results indicate that 2,4-DNP enhances the cytotoxic effect of cisplatin/etoposide on the LNCaP cell line.

In order to confirm the effect of the tested compounds on the cell cycle of LNCaP cells, the expression level of *CDKN1A* mRNA (a gene associated with cell cycle inhibition) was additionally determined (Fig. 4B). The incubation of the tested compounds alone with LNCaP cells for 24 h contributed to a significant increase in the relative CDKN1A mRNA level, compared to control cells. The highest increase was observed both for cisplatin and etoposide to level 30.53 ± 0.71 and 56.37 ± 1.37, respectively. In turn, simultaneous incubation of cells with uncoupler and tested drugs resulted

in a significant decrease in the relative *CDKN1A* mRNA level (over 6-times) for cisplatin and (almost 5-times) for etoposide used alone.

**Detection of apoptosis.** Apoptosis analysis indicated that nearly all the LNCaP cells treated with 2,4-DNP were early apoptotic. Following cisplatin and etoposide treatment, living cells predominated, as did cells in the early stages of apoptosis. In addition, a limited number of cells in the late stages of apoptosis were also detected. In turn, when the tested drugs were combined with 2,4-DNP, the number of cells in the late phase of apoptosis increased dramatically when compared to cells incubated with each compound alone, as well as the control (Fig. 5A, 5B).

**Oxidative stress.** In order to assess the potential effect of the tested compounds on the induction of oxidative stress, staining was performed with the CellROX dye. CellROX Green Reagent was used to evaluate ROS production in cells. Its bright green fluorescence is detected following oxidation by ROS and attachment to DNA (into the nucleus and mitochondria). In cells treated with 2,4-DNP, a small fluorescent signal originating from mitochondria was seen (Figure 6A). In the case of cisplatin and etoposide treatment intensive signal was noted mainly in the nuclei. The combined treatment resulted in the generation of the oxidative stress signal in both the nuclei and mitochondria.

The quantitative real-time PCR was used to examine the relative expressions of genes involved in oxidative stress defense. In compared to the control, the 2,4-DNP caused a statistically significant downregulation of all the examined genes (Figure 6B). In the case of *CAT* and *GPX1* genes the expression was barely noticeable, it fell below 1% compared to the control level and *NFE2L2* and *SOD2* gene expression lowered by over 70%. In contrast, the treatment of cells with chemotherapeutic agents used alone led to a significant increase in the expression of all tested genes. Incubation of LNCaP cells with cisplatin contributed to an increase of approximately 20%, 50%, and 80% in gene expression compared to control cells for *SOD2, CAT, GPX1* and *NFE2L2*, respectively. Especially in the cells treated with etoposide, the gene expression was elevated over 3 times for the *SOD2* gene and even higher 5.5 times for the *CAT* gene. The simultaneous incubation of cells with 2,4-DNP and cisplatin caused a sharp decrease in the expression of tested genes (the highest decrease in *CAT* and *GPX1)* in comparison to the control
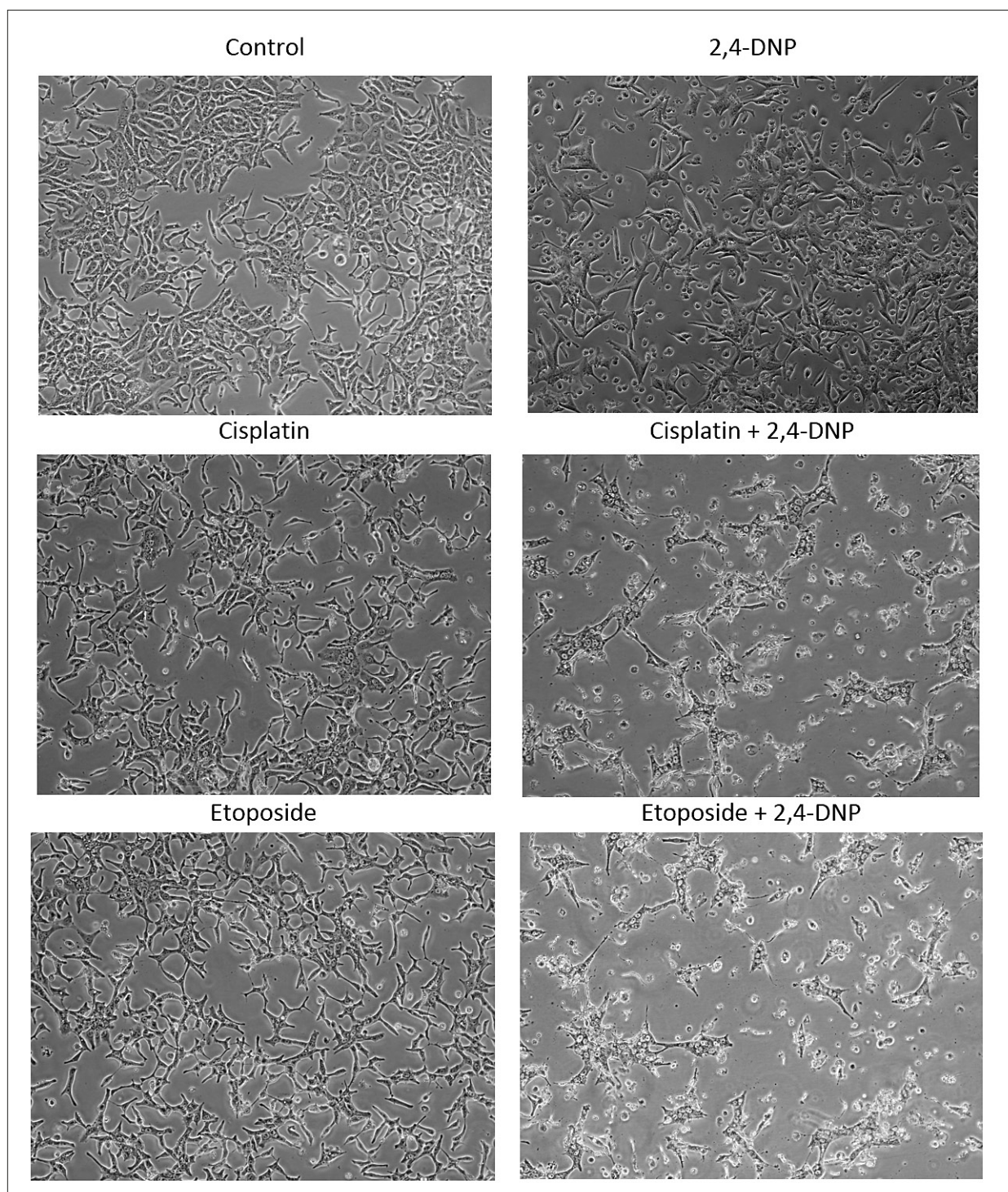
**Figure 3.** Changes in morphology of LNCaP cells. Cells were treated for 48 h with 2,4-DNP (100 µM), cisplatin (20 µM), etoposide (40 µM) or combined (2,4-DNP + CIS/ETO) (magnification ×100)

and cisplatin used alone. Similar results were noted in the case of the incubation of LNCaP cells with uncoupler and etoposide. The biggest downregulation was observed in *CAT* and *GPX1* genes. For the *NFE2L2* and *SOD2* genes, expression levels were downregulated compared to etoposide used alone over 2-times, while were elevated about 40% in comparison to the control.

*Expression of genes related to transporters associated with multi-drug resistance and DNA repair.* In order to evaluate the effect of the combination of cisplatin or etoposide with

2,4-DNP on transmembrane transporters, an analysis of the *ABCC1* and *ABCC4* genes was performed. Above mentioned genes encode proteins belonging to the ATP-binding cassette (ABC) transporter family (ABCC1- multidrug resistance protein 1/MRP1 and ABCC4- multidrug resistance protein 4/MRP4). These transporters are ATP-dependent and responsible for the efflux of drugs from cancer cells, thus contributing to treatment resistance. The analysis showed that 2,4-DNP caused statistically significant downregulation of both tested genes, about 60% in the case of the ABCC1
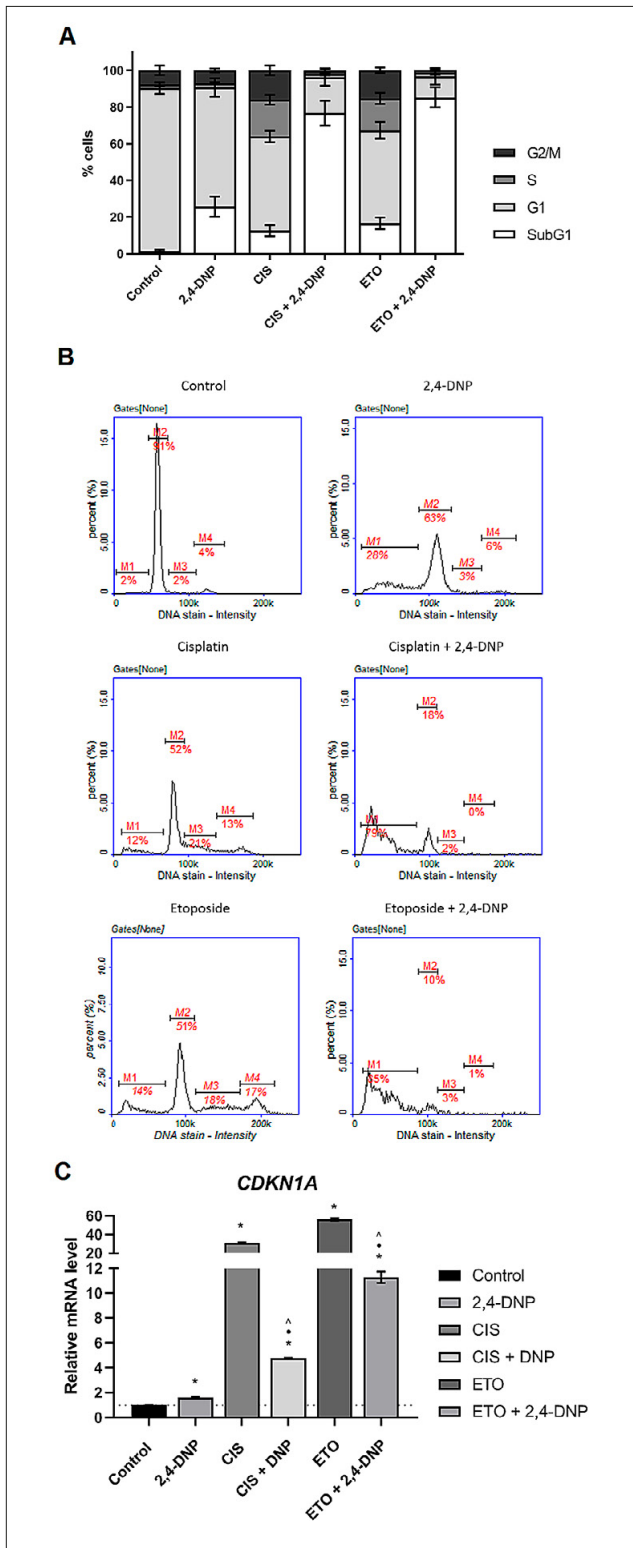
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**Figure 4.** Analysis of cell cycle of LNCaP cell lines. (A) Cells were treated for 48 h with 2,4-DNP (100 µM), CIS (20 µM), and ETO (40 µM), or combined (2,4-DNP +CIS/ETO). The values obtained from three independent experiments were presented as mean ± SD. (B) LNCaP cell cycle histograms representative of all repetitions of the experiment (M1—subG1, M2—G1, M3—S, M4—G2/M phase). C) Relative mRNA expression level of *CDKN1A* gene related to cell cycle inhibition. *BACT* and *RNA18SN5* were used as reference genes.
Results were calculated as RQ values and presented as mean ± SD. To compare more than two groups, the one-way analysis of variance (ANOVA) and *post hoc* multiple comparisons on a basis of Tukey's HSD test were used.
* $p < 0.05$ vs. Control; • $p < 0.05$ vs. 2,4-DNP; ^ $p < 0.05$ vs ETO,CIS
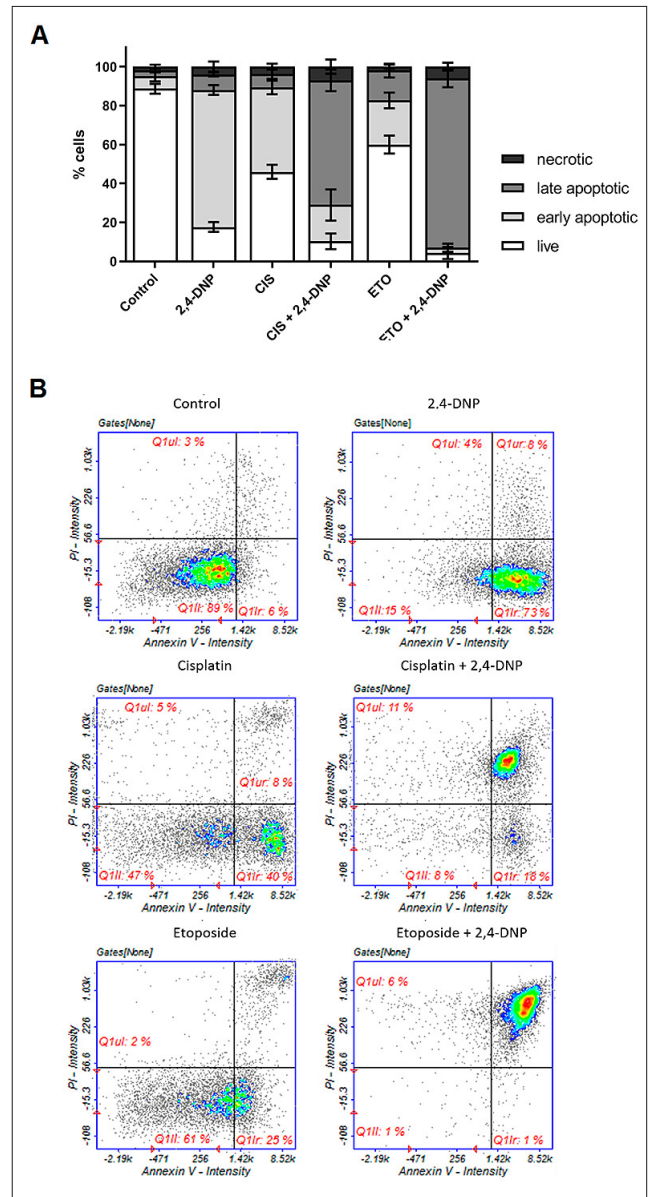


**Figure 5.** (A) Detection of cell apoptosis in LNCaP cells by image cytometry. The cells were treated with 2,4-DNP (100 µM), CIS (20 µM), and ETO (40 µM) or combined (2,4-DNP +CIS/ETO) for 48h.
Values obtained from three independent experiments are presented as mean ± SD. (B) Representative histograms (Q1ll – live; Q1lr – early apoptotic; Q1ur – late apoptotic, and Q1ul – necrotic cells).

gene as well as about 80% in the case of the ABCC4 gene, compared to control cultures (Figure 7A). Treatment of LNCaP prostate cancer cells with cisplatin or etoposide used alone contributed to a substantial increase in the expression of ABCC1 and ABCC4 genes, over 1.5 times for cisplatin and about 4 times for etoposide compared to control. The simultaneous treatment with 2,4-DNP and CIS or ETO led to the statistically significant downregulation of all the tested genes in comparison to chemotherapeutics used alone as well as to control cultures.

Next step of the study was assess the expression of genes associated with DNA repair. The mitochondrial uncoupler caused statistically significant decrease of the expressions of all tested genes. The expression in almost all cases fell below 85% as compared to the control. Analysis of *BRCA1* gene expression indicated that both chemotherapeutic agents
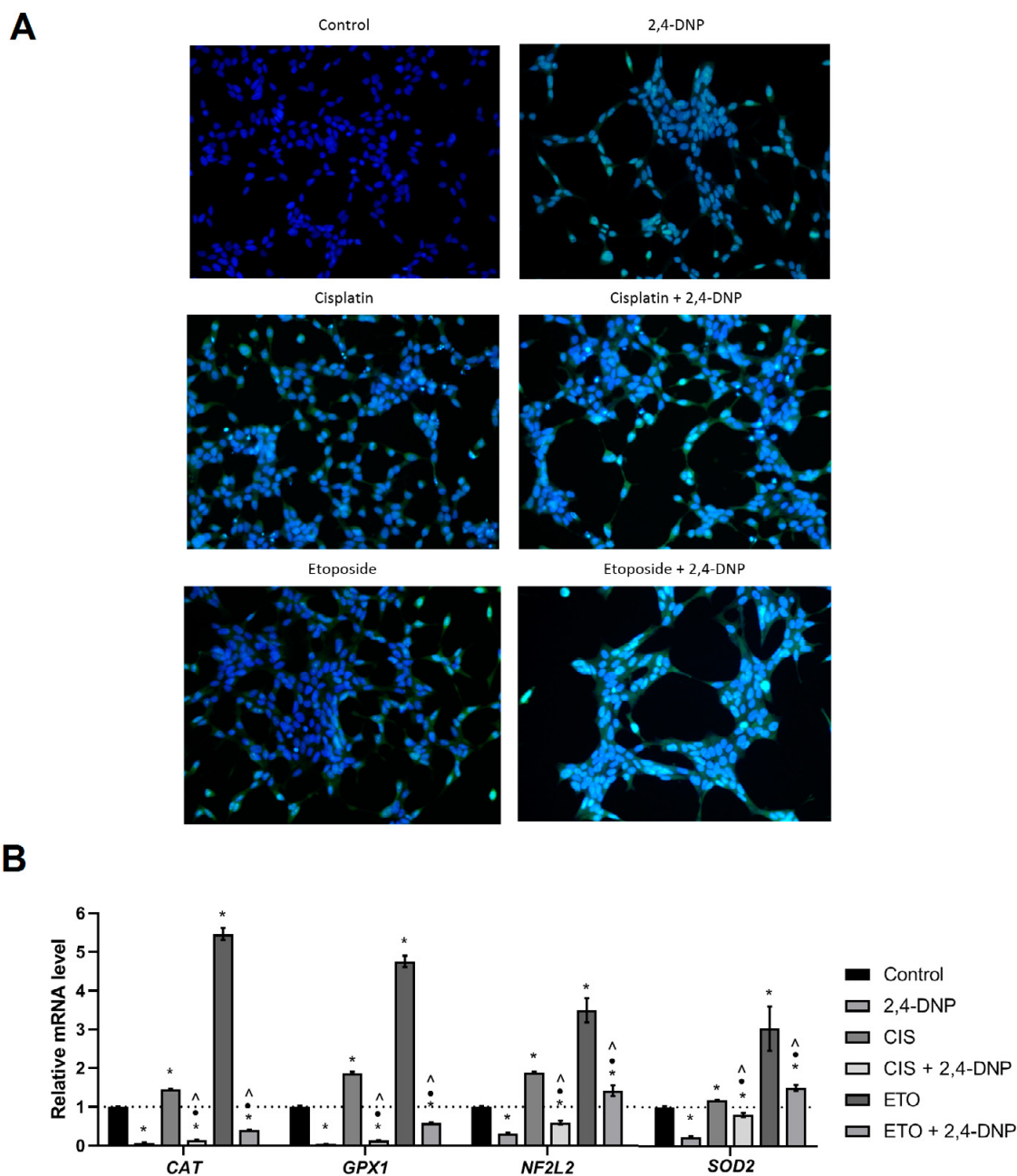
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**Figure 6.** (A) Detection of the oxidative stress in LNCaP cells using CellROX Green Reagent treated with 2,4-DNP (100 μM), CIS (20 μM), and ETO (40 μM) or combined (2,4-DNP +CIS/ETO) for 12 h. (B) Relative mRNA expression level of genes related to oxidative stress. *BACT* and *RNA18SN5* were used as reference genes. The results were calculated as RQ values and presented as mean ± SD. To compare more than two groups, the one-way analysis of variance (ANOVA) and post hoc multiple comparisons on a basis of Tukey's HSD test were used.
* $p < 0.05$ vs. Control; • $p < 0.05$ vs. 2,4-DNP; ^ $p < 0.05$ vs CIS, ETO.

used individually significantly reduced the expression of the mentioned gene (about 60%, and 80% for CIS and ETO, respectively) compared to control cultures. Moreover, the addition of 2,4-DNP resulted in a further statistically significant decrease in *BRCA1* expression in comparison to cisplatin or etoposide alone, respectively, and to the control cells. The *BRCA1* expression was barely noticeable, it fell about 99% compared to the control. Further studies revealed that CIS led to downregulation in the expression of all tested DNA repair genes (about 10–30%), in contrast to ETO which caused their significant upregulation (about 80–180%) compared to control cultures (Figure 7B). However, regardless of the used chemotherapeutic agent, the addition of the uncoupler resulted in a statistically significant decrease in the expression of tested genes compared to cells treated drugs alone.
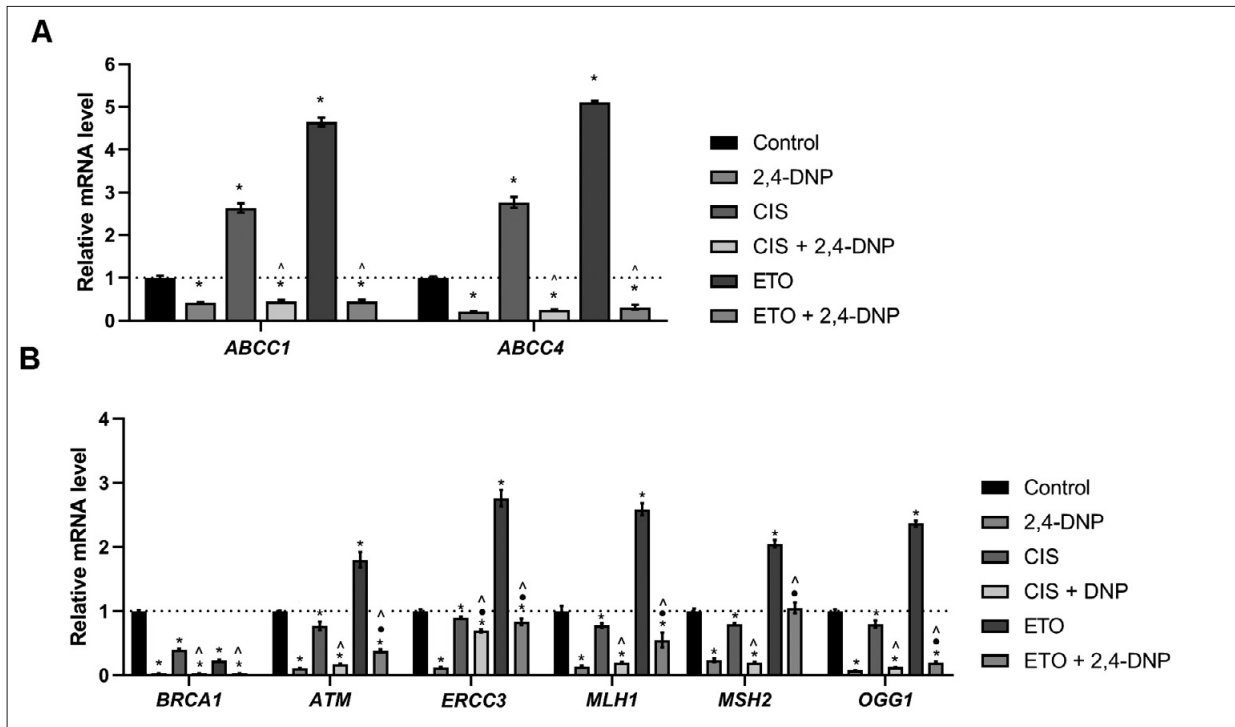
**Figure 7.** Relative mRNA expression level of genes related to (A) transporters associated with multi-drug resistance and (B) DNA repair. *BACT* and *RNA18SN5* were used as reference genes. The results were calculated as RQ values and presented as mean ± SD. To compare more than two groups, the one-way analysis of variance (ANOVA) and post hoc multiple comparisons on a basis of Tukey's HSD test were used.
* $p < 0.05$ vs. Control, • $p < 0.05$ vs. 2,4-DNP, ^ $p < 0.05$ vs CIS, ETO.

## DISCUSSION

The development of medical sciences in cancer biology allowed for the supposition that cancer is a multiclonal, heterogeneous disease, and this aspect must be considered in the decision-making process on the appropriate therapeutic regimen for an oncological patient. The differential sensitivity of individual cancer cells to the administered therapy may be closely related to the heterogeneity of cancer cells in the tumour. Because of the accumulation and/or development of additional mutations, malignant cell resistance develops as the disease progresses. The biggest problem for clinicians and scientists are small and undetectable in clinical conditions – clones of cancer cells that remain after treatment and are resistant to previously-used therapy. These subpopulations are often responsible for the recurrence of neoplastic disease, treatment resistance, and the presence of metastasis in other tissues and organs. To solve this problem, new ideas and a broader approach are needed to overcome the problem of drug resistance and therapeutic effectiveness. One of the strategies is based on combining classic anti-cancer drugs, such as chemotherapeutics, with other compounds of moderate toxicity in therapeutic regimens, which would improve the effectiveness of therapy. The choice of an additional factor should be based on knowledge about the biology of the tumour, including changes in the activity of cellular pathways, associated not only with rapid proliferation [4, 5]. One such change are the shifts in the metabolic phenotype of cancer cell associated with altered activity of energy-producing pathways. The phenomenon associated with these effect is called the Warburg effect and is based on the activation of glycolysis in cancer cells despite unrestricted access to oxygen [31].

Therefore, the disruption of mitochondrial activity in cancer cell populations, where the energy production is highly dependent on oxidative phosphorylation, may be one of the anticancer approaches. One example of the drugs widely used in medicine and have the ability to disrupt mitochondrial function, is metformin, which is used to treat diabetes. Many studies have indicated the anticancer potential of metformin based on a complex mechanism of action based, among others, on the inhibition of complex I of the electron transport chain. In studies conducted by Wheaton et al., metformin inhibited the proliferation and progression of colon cancer cells, depending on its ability to interfere with the function of the mitochondrial complex I [32, 33]. Despite the fact that compounds that inhibit mitochondrial complexes and disrupt mitochondrial function are usually characterized by low selectivity, scientists are still searching for new compounds, e.g. AG311, azoxystrobin and carboxyamidotriazole (CAI) [34–36].

A different treatment approach may be the combination of a compound affecting oxidative phosphorylation with chemotherapeutic agents used in routine anticancer therapy in cancer cells with a strong dependence on mitochondrial respiration. Such a compound may be 2,4-DNP which causes uncoupling of oxidative phosphorylation [8]. The aim of the current research was to investigate whether 2,4-DNP as a mitochondrial uncoupler sensitizes the prostate cancer cells to cytotoxic effect of cisplatin and etoposide.

Three prostate cancer cell lines (LNCaP, DU-145, PC-3) with established and diverse met-abolic phenotype were used in the study. The metabolic phenotype was confirmed in studies conducted by Higgins et al., and by the authors of the current study. Based on various parameters, the LNCaP cell line was found to be the most dependent on mitochondrial

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respiration due to the lowest level of lactate production and glucose uptake and high level of oxygen uptake. In addition, 2,4-DNP caused the greatest reduction in ATP level in LNCaP prostate cancer cells compared to the other prostate cancer cell lines (DU-145, PC-3), and led to the greatest decrease in LNCaP cells viability [20, 21].

The performed MTT assay showed that the simultaneous incubation of 2,4-DNP with cisplatin or etoposide enhanced the cytotoxic effect of the chemotherapeutic agent only in LNCaP cells. The obtained results were confirmed by microscopic observation, cell cycle analysis and apoptosis detection. Etoposide and cisplatin used individually caused a cytostatic effect characterized by an increased population of LNCaP cells in S and G2/M phase. The occurrence of this effect is typical for the used chemotherapeutic agents and is consistent with other research studies [37–39]. The addition of 2,4-DNP to cisplatin or etoposide results in the abolition of the cytostatic effect and the appearance of a strong cytotoxic effect in LNCaP cells. Cell cycle analysis was also confirmed at the molecular level by examining the expression of the CDKN1A gene, which encodes the p21 protein responsible for regulation of cell proliferation by inhibiting the cell cycle through the cyclin kinase pathway [40]. A significant decrease in gene expression in the case of a combination of a chemotherapeutic agent with 2,4-DNP in relation to the drug administered alone indicated a reduction in the cytostatic effect.

All the above-mentioned results may indicate that 2,4-DNP does not intensify the main mechanism of action of cisplatin and etoposide, which are considered to be DNA alkylation and topoisomerase II inhibition, respectively [14, 15]. It is highly probable that the enhancement of the therapeutic effect of chemotherapeutic agents by 2,4-DNP relies on a different mechanism of action.

In further research, the authors of the current study assessed whether oxidative stress and redox imbalance may be a process involved in enhancing the effect of chemotherapeutic agents by 2,4-DNP on LNCaP cells. As is well known, the main mechanism of action of 2,4-DNP is the uncoupling of oxidative phosphorylation, which leads to the disruption of basic mitochondrial functions. Disruption of ATP formation by 2,4-DNP in mitochondria contributes to a serious redox imbalance. 2,4-DNP causes a slight induction of oxidative stress as indicated by CellRox fluorescent dye staining. The current study shows that 2,4-DNP strongly contributes to the redox imbalance. Gene expression analysis revealed that 2,4-DNP strongly down-regulated all genes related to antioxidant defence. In addition, previous research by the authors has shown the impact of the uncoupler on redox balance, as evidenced by a decrease in the level of GSH which is an essential component of the antioxidant defence system and an increased amount of AP sites, which indicate oxidative DNA damage [22].

Studies performed by Yu et al. and Shin et al. indicated that both cisplatin and etoposide can induce oxidative stress [16, 41], results confirmed in the research by the authors of the current srtudy. Both chemotherapeutics used alone caused not only oxidative stress in LNCaP cells (CellRox staining), but also statistically significant increased in the expression of genes related to defence against oxidative stress. This study indicates that simultaneous incubation of cells with 2,4-DNP and cisplatin or etoposide do not lead to an increase in the level of oxidative stress, but to the abolition of the LNCaP

cell`s ability to defend against the toxic effect of free radicals. Analysis of gene expression showed that the addition of 2,4-DNP results in a significant decrease in the expression of antioxidant defence genes, compared with single agents used alone. The lack of antioxidant defence caused by 2,4-DNP and the induction of oxidative stress by etoposide and cisplatin may be one of the proposed mechanisms responsible for the increased cytotoxic effect in LNCaP cells.

A major role in the resistance of prostate cancer cells to chemotherapy is played by transmembrane transporters belonging to the ATP-binding cassette (ABC) efflux transporters family (MRP/ABCC) i.a. MRP1 (*ABCC1* gene) and MRP4 (*ABCC4* gene) [42–44]. The over-expression of these proteins in cancer cells is responsible for the efflux of the chemothera-peutic drugs from the inside of the cancer cell through the cell membrane using the energy derived from ATP hydrolysis [45]. Analysis of the expression of *ABCC1* and *ABCC4* genes revealed that both cisplatin and etoposide cause upregulation of the expression of both tested genes. However, incubation of LNCaP cells with 2,4-DNP alone, as well as simultaneous incubation with a chemotherapeutic agent and an uncoupler, result in a dramatic decrease in the expression of genes encoding MRP1 and MRP4 multidrug resistance proteins. It is likely that the inhibition of ATP formation by 2,4-DNP in LNCaP cells results in a lack of the energy necessary for the functioning of ABC transporters. Consequently, the chemotherapeutic agent may accumulate in cancer cells, thus enhancing the cytotoxic effect. Enhancement of the therapeutic effect of cisplatin and etoposide by 2,4-DNP occurred only in the case of the LNCaP line, which may be due to the fact that these cells obtain energy mainly in the process of oxidative phosphorylation. Uncoupling of this process by 2,4-DNP resulted in a significant decrease in ATP production [21; however, elucidation of the role of ABC transporters requires further in-depth research. In addition, analysis of the expression of repair genes showed that the simultaneous incubation of prostate cancer cells with 2,4-DNP and chemotherapeutic drugs reduces the ability of cells to repair the resulting DNA damage. Thus, the increased effects of cisplatin and etoposide under the influence of 2,4-DNP are responsible for the disturbed redox balance, the reduced ability of cells to repair DNA, and the oxidative metabolic phenotype of prostate cancer cells.

## CONCLUSIONS

Despite the fact there have been significant advances in anticancer therapy, they have not minimized the burden of the disease and cancer remains a major global health issue. The discovery of novel treatments for cancer, including prostate cancer, is therefore of paramount importance. One of the novel approaches might be to sensitize cancer cells with a certain metabolic profile by employing drugs that disrupt multiple energy metabolism pathways.

The enhanced effect of 2,4-DNP and cisplatin/etoposide on LNCaP prostate cancer cells with an oxidative phenotype, as demonstrated in this study, might serve as a starting point for future research into various types of malignancies and substances.

**Data availability statement.** Data presented in this study are available on request from the corresponding author.

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