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The role of oxidative stress in the cooperation of parthenolide and etoposide in HL-60 cells

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Abstract: Background: The aim of this study was to determine the effect of sesquiterpene lactone parthenolide on the cytotoxic and pro-oxidative effects of etoposide in HL-60 cells.
Methods: Cytotoxic effects were determined by incubation of HL-60 cells with various concentrations of examined compounds and combinations thereof, which were then stained with propidium iodide and analyzed using a flow cytometer. To determine the role of oxidative stress in the action of the compounds, co-incubation with N-acetyl-l-cysteine (NAC) and parthenolide and/or etoposide was used and the level of reduced glutathione (GSH) was detected.
Results: Parthenolide significantly enhanced the cytotoxic and pro-apoptotic effects of etoposide. However, in most cases of the combinations of parthenolide and etoposide, their effect was antagonistic, as confirmed by an analysis using the CalcuSyn program. The examined compounds significantly reduced the level of GSH in HL-60 cells. Combination of etoposide at a concentration of 1.2 μM and parthenolide also significantly reduced GSH level. However, in the case of a combination of etoposide at a concentration of 2.5 μM with parthenolide, a significant increase in the level of GSH was obtained compared to compounds acting alone. This last observation seems to confirm the antagonism between the compounds tested.
Conclusions: Parthenolide did not limit the cytotoxic effect of etoposide in HL-60 cells even in the case of antagonistic interaction. If parthenolide does increase GSH levels in combination with etoposide in the normal hematopoietic cells, it could protect them against the pro-oxidative effects of this anti-cancer drug.

Keywords: parthenolide, etoposide, acute myeloid leukemia, GSH, oxidative stress, flow cytometry.

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Introduction

Despite the great progress that has been made in the implementation of modern anti-cancer therapies, classical cytotoxic drugs are still used on a large scale. Cytostatic drugs not only cause many adverse effects but also have a relatively low treatment efficacy for such cancers as acute myeloid leukemia (AML). The incidence of AML increases significantly after 60 years of age, and the 5 year survival rate in this age group is around 5% [1]. AML encompasses a number of types of leukemias derived from stem cells or progenitors of a myeloid lineage and this is a very heterogenic disease in cytogenetics [2, 3], which makes it difficult to develop an effective treatment.

In the light of the above, studies on improving the effectiveness and limiting the side effects of classic cytostatic drugs are important. A lot of works on the synergistic action of anti-cancer drugs and certain plant components have been published [4–6]. A significant problem in the use of compounds isolated from plants is often their low bioavailability. One such compound with promising cytotoxic activity in leukemic cells is sesquiterpene lactone parthenolide [7]. The action of this compound is important, because it has cytotoxic activity in leukemic stem cells, which are the most resistant to therapy, while not having a significant effect in normal hematopoietic stem cells [8]. This dualistic effect in cancerous and normal cells is known for many plant compounds having antineoplastic activity [9, 10]. However, increased cytotoxicity toward cancer stem cells is a less common feature among plant-derived components [11]. The main problem with the use of parthenolide is the instability of its molecule and its low bioavailability, as demonstrated in clinical studies [12]. Currently, a derivative of parthenolide which retains the action characteristic of the parent compound and with better bioavailability is being tested [7]. Therefore, analyzing the action of parthenolide is important in the context of its derivative, which has the chance to be used in clinical practice.

The source of parthenolide are the leaves of *Tanacetum parthenium*, a perennial from the Asteraceae family [13]. The antitumor effect of parthenolide is attributed to the presence of a lactone ring and, above all, to an α-methylene-γ-lactone moiety, which has alkylating properties [14, 15]. An important element of the action of parthenolide is its ability to display Michael-type nucleophilic addition reactions with-SH groups [16].

Its antineoplastic activity mainly consists of inhibiting NF-kB activation, blocking signal transducers and activators of transcription 3 phosphorylation (STAT3), and also exerting pro-oxidative action in tumor cells, by inhibiting glutathione metabolism [8, 17].

Etoposide is a classic cytotoxic drug used to treat certain solid tumors as well as hematological cancers, including acute myeloid leukemia In the case of AML, etopo-

side is used in some protocols during induction or consolidation therapy [18, 19]. The mechanism of action of etoposide consists of fixing DNA-topoisomerase II cleavable complexes, which leads to double-stranded DNA breaks in proliferating cells followed by their apoptosis [20]. Its effectiveness in therapy limits its side effects, such as myelosuppression and granulocytopenia. Considering that parthenolide may increase the effect of some compounds with antitumor activity [21, 22], we have focused on the analysis of the interaction between etoposide and parthenolide in HL-60 cells.

Materials and Methods

HL-60 cell culture and treatment

HL-60 cell line (American Type Culture Collection, ATCC, European branch in UK) was propagated in RPMI-1640 medium with 10% FBS at 37°C in a 95% humidified atmosphere and in 5% CO_2. Antibiotics were not added to the culture medium. Cells were incubated with parthenolide and/or a cytostatic drug for 24 hours in 24-well culture plates (0.5×10^6 cells/ well). Compounds were dissolved in DMSO, diluted in PBS and stored at −20C. The maximum concentration of DMSO in culture medium was 0.7%. Control cells were incubated with DMSO and without the investigated compounds. In order to investigate the role of oxidative stress in the action of PTL and ETO, cells were incubated simultaneously with 6 mM N-acetyl-l-cysteine (NAC) and tested compounds.

Investigation of cytotoxicity and analysis of combination index

Dead cells were stained with propidium iodide according to instructions developed by the R&D Systems Flow Cytometry Laboratory. Briefly, HL-60 cells were treated with the compounds to be investigated, then they were washed (230 g, 5 min), resuspended in 500 μL PBS and stained with 10 μL PI staining solution (10 μg/mL). Incubation lasted 15 minutes and was conducted in the dark, at room temperature. Thereafter, the cells were immediately collected and analyzed with a flow cytometer (LSRII, BD Biosciences Immunocytometry Systems, San Jose, CA, USA) using blue and red lasers. PI-width versus PI-area calculations were used to exclude doublets anddebris.

Both the combination index (CI) and the multiple drug-effect equation developed by Chou-Talalay, were applied; thus 0.4< CI <0.6 indicates synergism, 0.6< CI <0.8 moderate synergism, 0.8< CI <0.9 slight synergism, CI >0.9 an additive effect, and CI >1.1 antagonism.

Study of apoptotic and necrotic cells

In order to identify apoptotic cells, we used double staining with annexin-V-APC and propidium iodide. We used the dyeing conditions (concentration of dye and time of incubation) recommended by the manufacturer. The cells were immediately analyzed with a flow cytometer (LSRII, BD Biosciences Immunocytometry Systems).

The percentages of early (Annexin V-APC-positive/PI-negative), late (Annexin-APC-positive/PI-positive) and necrotic (Annexin-APC-negative/PI-positive) were determined using FACSDiva software (Becton Dickinson). The analysis was performed on 10 000 single cells.

Analysis of GSH level

The cells were incubated for 5 minutes with 2 µl of 37% (v/v) HCl, gently vortexed, sonicated and centrifuged at 8,000 × *g*, 4°C for 15 min. The total GSH content in HL-60 tumor cell lines was measured using the standard method described by Tietze [23]. The following were used for this determination: phosphate buffer (NaH_2PO_4) 0.2 mol/L at pH = 8.2, DTNB 10 mmol/L dissolved in 100 ml of phosphate buffer 0.2 mol/L at pH = 8.2, and trichloroacetic acid (TCA) 2.5%. GSH 0.001 mol/L at concentrations of 10–100 µmol/L was used as standard. Reagents for the assays were purchased from Sigma Chemical Co., St. Louis, MO.

The working solution was prepared on the day of the assay and contained 150 µl of 0.2 mol/L phosphate buffer at pH = 8.2; 40 µl 10 mmol/L DTNB and 110 µl supernatant (deproteinized cell pellet). The supernatant was placed in wells of a 96-well plate in ice and a working solution was added, and a stopwatch was immediately activated after the working stream was added. The absorbance of the resulting complex was measured at a wavelength of λ max = 412 for the blank (2.5% TCA) after 5 min from the addition of the working time. Absorbance was measured at room temperature. The readings were taken on a FLUOstar Omega spectrophotometer (BMG Labtech, Germany).

Protein determination

Total protein level measurements were performed three times in cell suspensions with bicinchoninic acid (BCA) according to the manufacturer's instructions (Sigma-Aldrich, USA). The standard used was bovine serum albumin (BSA). The BCA method is based on the reduction of Cu^{2+} to Cu^{+}, where in sequence Cu^{+} ions react with BCA acid, leading to the formation of a violet color. Absorbance is measured at a wavelength of λ max = 562 nm. Measurements were made for reference, blank and test samples. 25 µl of the standard solution/test/blank solution was mixed with

200 µl of the working solution. After adding all the reagents, the plate was sealed with a protective film to protect against evaporation. The reagents were incubated for 30 min at 37°C. The plates were then cooled to room temperature and the absorbance of the samples was measured at a wavelength of λ max = 562 nm at 37°C. The calibration curve was determined, and the protein concentration was determined from the linear regression equation expressed in g/L.

Statistical analysis

The U Mann–Whitney test or one-way analysis of variance (ANOVA) and the Tukey post-hoc test were used in the analysis of the statistical significance between groups. All data are shown as means ± standard deviation of the mean.

In the case of GSH analysis, a comparison of the values of quantitative variables in three or more groups was performed by ANOVA (if the variable had a normal distribution in these groups) or the Kruskal–Wallis test (in other cases). After the detection of statistically significant differences, a post-hoc analysis was performed with the Fisher's LSD test (in the case of normality of distribution) or Dunn's test (in the absence of normality), in order to identify statistically different groups. The normality of the variable distribution was examined using the Shapiro–Wilk test. The significance level of 0.05 was assumed in the analysis. So, all p values below 0.05 were interpreted as indicating significant dependencies. The analysis was performed in the R program, version 3.5.1.

Results

Cytotoxic and proapoptotic activity of the tested compounds and their combinations

Parthenolide exerted a significant cytotoxic activity in HL-60 cells starting from a concentration of 2.5 µM after 24 hours of incubation in comparison to the control (Fig. 1A). The use of parthenolide and etoposide in combinations of different concentrations indicates antagonism between them, which was demonstrated in most combinations (Fig. 1B, 1C; 2). Only in the case of etoposide at the concentration of 1.2 µM and parthenolide in the concentration of 5 µM was synergism in cytotoxic activity obtained, because the combination effect exceeded the sum of the action of the two separate components (Fig. 1B, 2B). A twice higher concentration of etoposide (2.5 µM) with parthenolide at the concentration of 5 µM had an antagonistic effect (Fig. 1B, 2C). In the case of parthenolide at 2.5 µM and etoposide at 1.2 µM, antagonism was also present (Fig. 1A).

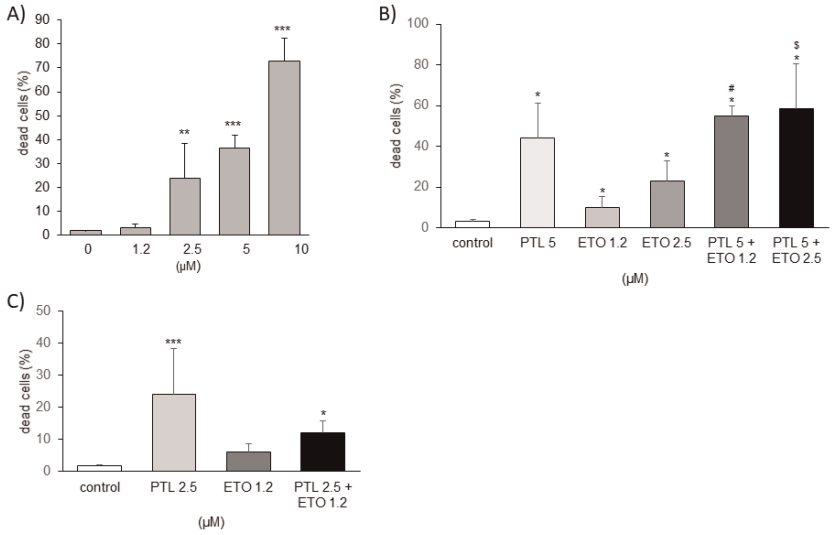

Fig. 1. The quantitative analysis of dead HL-60 cells after 24 hours incubation with the examined compounds. (**A**) The influence of the different concentrations of parthenolide (PTL) on the amount of dead cells. (**B, C**) The influence of combination of PTL and etoposide (ETO) (**A–C**). *p <0.05 vs. control, **p <0.01, ***p <0.001, #p<0.001 vs. ETO 1.2 μM, $p <0.001 vs. ETO 2.5 μM.

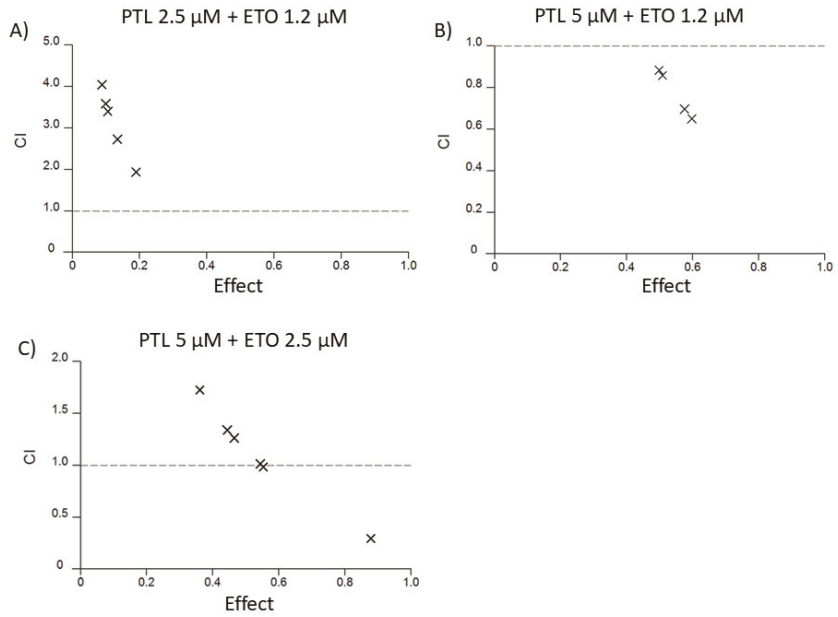

Fig. 2. Analysis of interaction between etoposide (ETO) and parthenolide (PTL). Monte Carlo simulation (**A–C**). x — combinations of compounds, CI — combination index; 0.4< CI <0.6 synergism, 0.6< CI <0.8 moderate synergism, 0.8< CI <0.9 slight synergism, CI >0.9 additive effect, CI >1.1 antagonism.

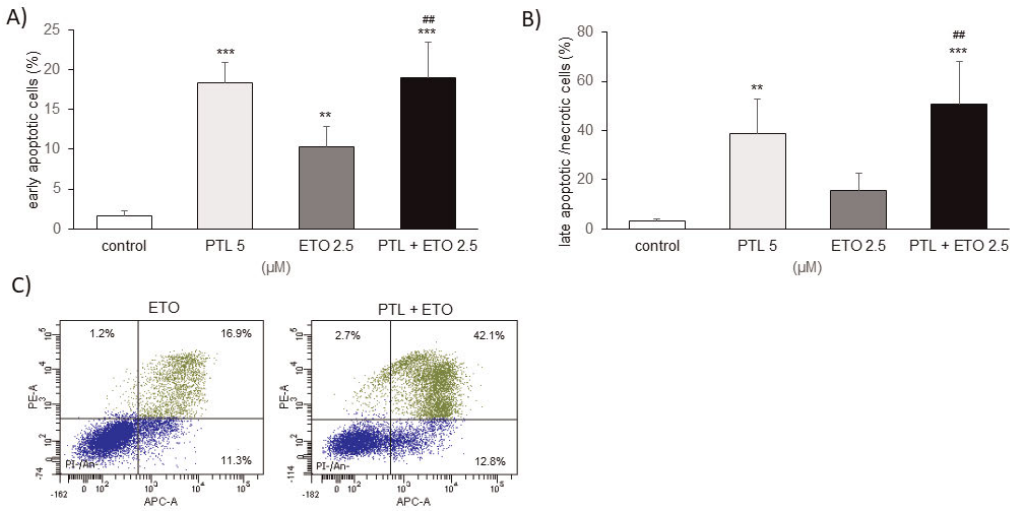

Fig. 3. Apoptosis of HL-60 cells after 24 hours incubation with parthenolide (PTL), etoposide (ETO) and combination of two examined compounds. (**A, B**) The percentage of early-, late-apoptotic and necrotic HL-60 cells. **p <0.01, ***p <0.001 vs. control, ##p <0.01 vs. ETO2.5 μM. (**C**) Representative dot plots of annexin-V vs. propidium iodide staining.

Parthenolide at a concentration of 5 μM significantly increased the number of early-and late-apoptotic cells; while, in the case of etoposide at 2.5 μM, a significant increase only in early apoptosis after 24-hour incubation was observed in comparison to the control (Fig. 3A, B). In the case of a combination of the tested compounds, a significant increase in early and late apoptosis compared to etoposide alone was observed, but the effect was not significant in comparison to parthenolide alone (Fig. 3).

Analysis of the role of oxidative stress in the action of the examined compounds

The addition of antioxidant NAC to cells incubated with parthenolide abolished the cytotoxic and pro-apoptotic effects of this compound almost to the control level (Fig. 4A, 4B). NAC, however, did not significantly affect the cytotoxic effect of etoposide (Fig. 4A). The cytotoxic effect of the combination of the two compounds significantly decreased after the simultaneous use of NAC almost to the level of action of etoposide alone (Fig. 4A, 4C).

Both etoposide and parthenolide significantly decreased GSH levels in HL-60 cells compared to the controls (Fig. 5A). The combination of a lower concentration of etoposide (1.2 μM) with 5 μM parthenolide significantly reduced the level of GSH compared to the control (p <0.001) and this effect was comparable to the action of the

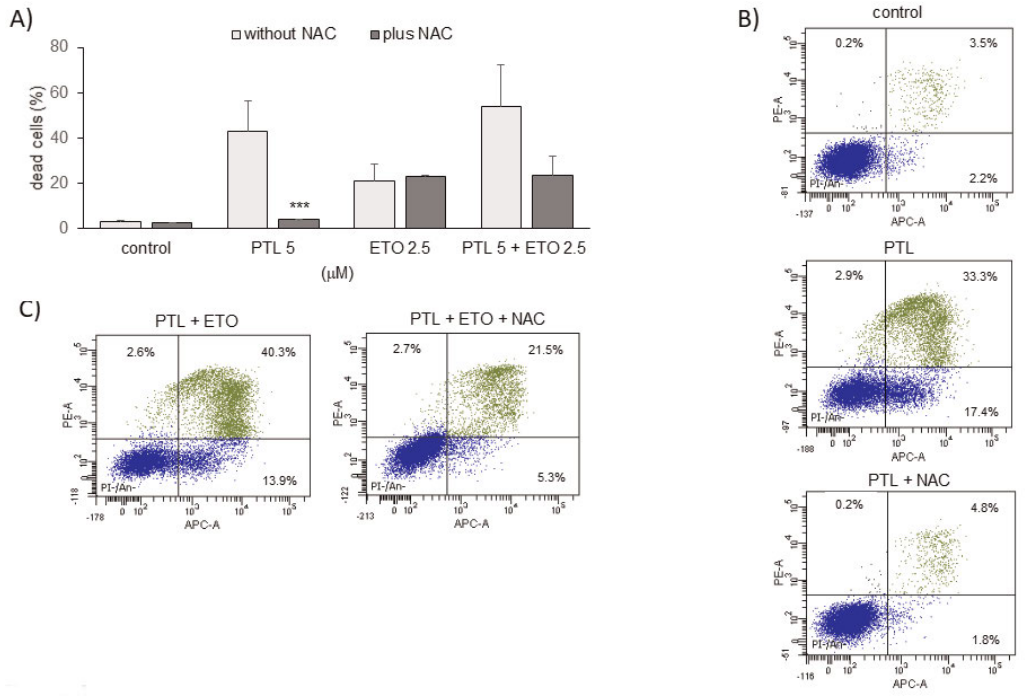

Fig. 4. The influence of N-acetyl-l-cysteine (NAC) on the effect of parthenolide (PTL), etoposide (ETO) and combination of two examined compounds in HL-60 cells after 24 hours of incubation. (**A**) The percentage of dead HL-60 cells. ***p <0.001 vs. control. (**B, C**) Representative dot plots of annexin-V vs. propidium iodide staining.

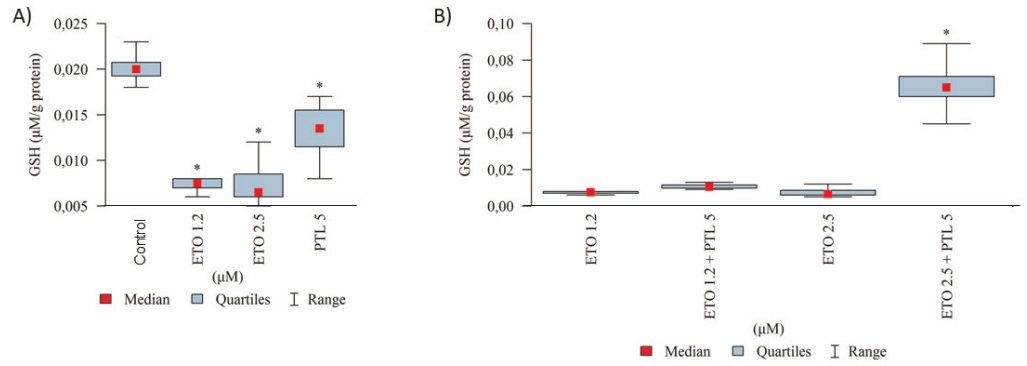

Fig. 5. The level of GSH in HL-60 cells after 24 hours incubation with the examined compounds. (**A**) The influence of parthenolide (PTL) and etoposide (ETO). (**B**) The influence of combination of PTL and ETO compared to ETO alone. *p <0.001 vs. control.

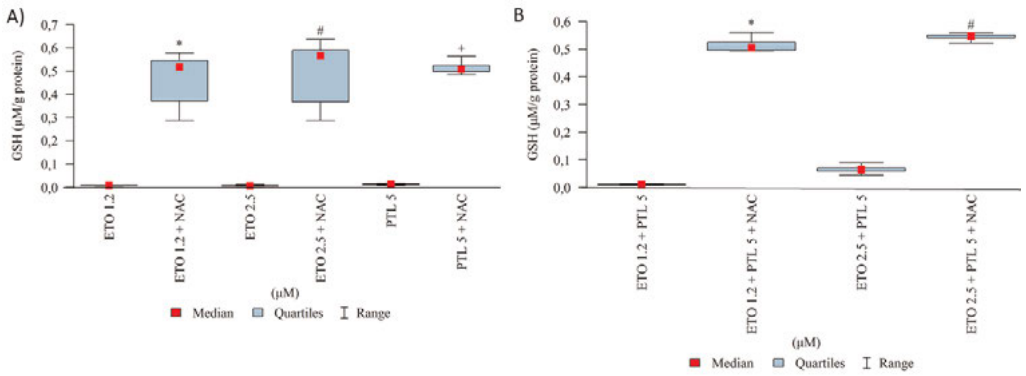

Fig. 6. The influence of N-acetyl-l-cysteine (NAC) on the level of GSH in HL-60 cells after 24 hours incubation with the examined compounds. (**A**) The influence of NAC plus PTL and NAC plus ETO. (**B**) The influence of NAC plus combination of ETO and PTL. *p <0.001 vs. ETO 1.2 μM, ETO 1.2 + PTL 5 (μM); #p <0.001 vs. ETO 2.5 μM, ETO 2.5 + PTL 5 (μM); +p <0.001 vs. PTL 5 μM.

single etoposide used (Fig. 5). An inverse effect was obtained after using a two times higher concentration of etoposide (2.5 μM) in combination with 5 μM of parthenolide. Here, a significant increase in GSH level was observed in comparison to etoposide alone (Fig. 5B). The addition of NAC to incubation with one of the examined compound or the combination of two compounds significantly increased the level of GSH compared to incubation without NAC (Fig. 6A, 6B).

Discussion

Parthenolide is a compound whose mechanism of action is studied extensively due to its ability to kill CD34+ leukemia stem cells. Its derivative with an increased bioavailability of dimethylamino parthenolide (DMAPT) can be employed in anti-cancer therapy. Less attention is paid to the potential interactions of parthenolide with classic cytotoxic drugs.

One of the mechanisms of action of parthenolide in cancer cells is the induction of oxidative stress. According to our research and studies by other authors [7, 17], induction of oxidative stress is the main mechanism of parthenolide anti-leukemic action. In this study, the use of NAC almost completely abolished the cytotoxic action of parthenolide in HL-60 cells. In addition, parthenolide significantly reduced GSH levels, and this effect was reversed by NAC. These results correlate with those of other studies demonstrating the inhibitory effect of parthenolide on GSH, as well as on key glutathione metabolism enzymes, such as glutamate-cysteine ligase (GCL) and glutathione peroxidase 1 (GPX1) [7].

In the search for new anti-cancer strategies, attention has recently been focused on modifying the metabolism of cancer cells, including oxidative metabolism. Many studies show that cancer cells having elevated levels of free radicals are more susceptible to the cytotoxic effects of pro-oxidants compared to non-cancerous cells. Combining a pro-oxidative strategy with the conventional cytotoxic therapy against cancer cells may provide a new tool for more effective treatment of AML.

Etoposide, a cytostatic drug used in some AML treatment protocols, can also cause the grow in the amount of free radicals in cancer cells. In these and our own previous studies, etoposide does not act cytotoxically through oxidative stress, because NAC did not affect the percentage of dead cells induced by this anticancer drug in HL-60 cells [24].

The synergistic action of the two tested compounds was obtained only at the lowest concentration of etoposide of 1.2 μM. This concentration of etoposide may be present in the patients' blood serum [25]. The question arises whether the high level of oxidative stress generated by the combination of 1.2 μM etoposide and 5 μM of parthenolide could be responsible for the intensification of cytotoxic action in comparison to single acting compounds? However, a higher concentration of etoposide (2.5 μM) resulted in most of the combination with parthenolide antagonistic or additive interaction. Also, the combination of parthenolide at 2.5 μM with 1.2 μM etoposide led to antagonism in terms of cytotoxicity. The antagonistic effect of the combination did not significantly reduce the cytotoxic effect of the anticancer drug alone but it significantly reduced its pro-oxidative effect in leukemic cells. The use of the combination, however, weakened the pro-oxidative activity of parthenolide most severely. It can be assumed that the obtained effect corresponds to the competition between etoposide and parthenolide for the binding site in myeloperoxidase (MPO). Both compounds are oxidized by MPO, which participates in their pro-oxidative activity. The MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) or small interfering RNA (siRNA) specific for MPO significantly reduced the pro-oxidative and apoptotic effects of parthenolide in leukemic cells with constitutively high MPO activity [26]. Etoposide, as a MPO substrate, is oxidized to phenoxyl radicals responsible for pro-oxidative activity in hematopoietic stem cells [27]. Etoposide at the concentration of 2.5 M can compete with parthenolide as a substrate for MPO that leads to antagonism between these compounds. On the other hand, etoposide at the lower concentration of 1.2 μM may compete less effectively with PTL for binding to MPO, and other mechanisms, such as inhibition of NF-kB by parthenolide, may participate in synergistic action of these compounds. As demonstrated in other studies, NF-kB increases the expression of Bcl-2 and Bcl-xL mRNA, which are responsible for inhibiting the activation of etoposide-induced caspase [28].

The literature data show that AML CD34+ stem cells have a deregulated glutathione metabolism. A low level of glutathion is found in these cells and the ratio

of GSH to glutathione disulfide (GSSG) is changed [17]. HL-60 cell line derived from human promyelocytic leukemia used in the present study has active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a source of reactive oxygen species (ROS), and is considered a good model for the study of pro-oxidants [29]. In such cells with an elevated level of oxidative stress, parthenolide may additionally intensify this deregulation of homeostasis. Using the metabolic shift toward oxidative metabolism, parthenolide can be an effective cytotoxic agent in all cancer cell populations, including stem cells. The mechanism of its pro-oxidative action may correspond to the lactone moiety, thanks to which parthenolide may bind to thiol groups in the cysteine glutathione sites and enzymes regulating its metabolism [17, 30].

This study shows that, due to antagonism, the combination of parthenolide with etoposide may not be of practical use in promyelocytic leukemia cells. Parthenolide did not significantly reduce the cytotoxic effect of etoposide in leukemic cells, but decreased its pro-oxidative activity. If a similar effect occurs in normal hematopoietic cells, parthenolide may protect them from the pro-oxidative effects of the phenoxyl radicals of etoposide.

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Conflict of interest

None declared.

Abbreviations

ABAH — 4-Aminobenzoic Acid hydrazide
AML — acute myeloid leukemia
GSH — reduced glutathione
MPO — myeloperoxidase
NAC — N-acetyl-l-cysteine

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