MEDICAL UNIVERSITY OF LODZ

International Doctoral School Medical University of Lodz

Assessment of the possibility of using endogenous lipids in anti-cancer therapy in the mechanism dependent on endoplasmic reticulum stress

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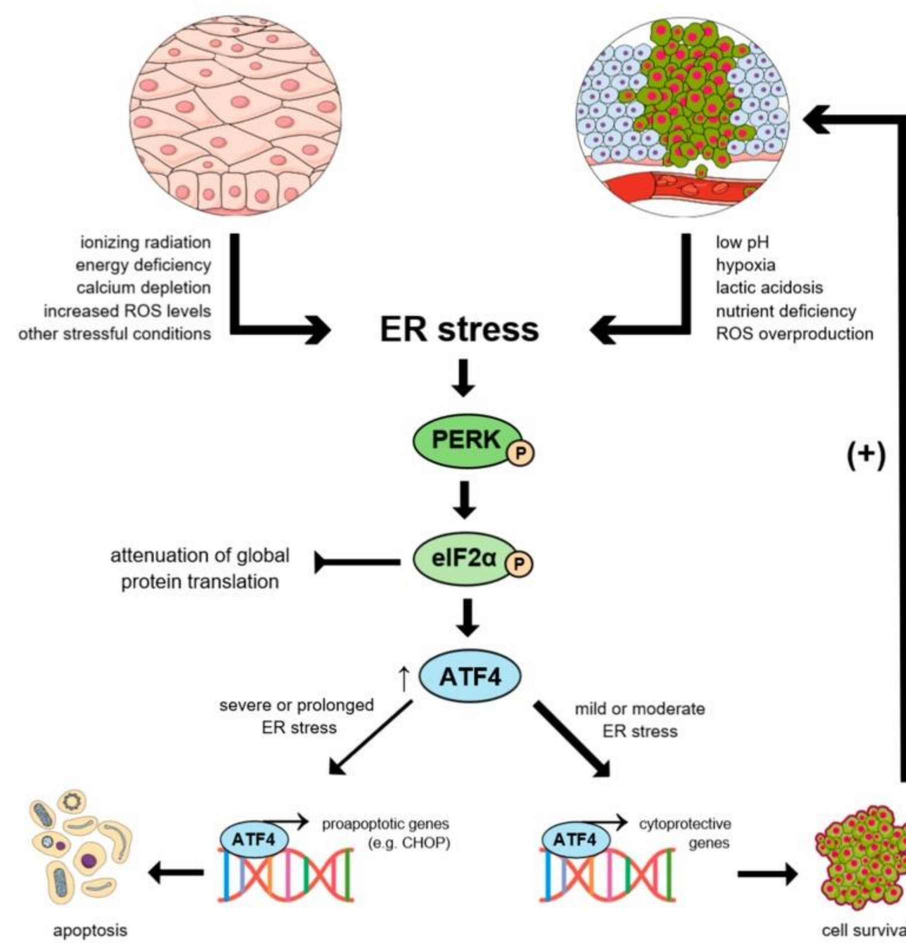

Figure 1. Unfolded Protein Response (UPR)

Introduction:

The uncontrolled growth of cells as well as inactivation of apoptosis are strongly connected with cancer development and progression. Abnormal proliferation of tumor mass results in structural malformations and impairment of angiogenesis that rapidly evoke a hypoxic state in cancer cells environment inducing the Endoplasmic Reticulum (ER) stress, which subsequently activates the Unfolded Protein Response (UPR) signaling pathway – figure 1.

There is an ample of evidence that cellular alterations on the molecular level, leading to cancer development and progression, are directly evoked upon ER stress-dependent activation of PERK. Activated PERK triggers phosphorylation of its main substrate namely the Eukaryotic initiation factor 2 alpha (eIF2α). Above-mentioned event triggers downregulation of global protein synthesis and, on the other hand, enhanced translation of only selected proteins such as Activating transcription factor 4 (ATF4) characterized by a dual role: pro-adaptive and pro-apoptotic. The pro-adaptive role of the ATF4 is closely connected with the adaptation of cancer cells to adverse, hypoxic conditions, whereas upon severe and long-termed ER stress conditions ATF4 induces a pro-apoptotic branch of the UPR via upregulation of cell death-related genes including CCAAT-enhancer-binding protein homologous protein (CHOP).

The main aim of the present study was to evaluate the effectiveness of the small-molecule PERK inhibitor in an *in vitro* cellular model of human lung carcinoma.

Materials and methods:

The anti-tumor properties of previously selected small-molecule PERK inhibitor were examined on commercially available human lung carcinoma epithelial cel line A549 and the normal human pulmonary fibro-blasts HPF.

The cytotoxicity of the selected PERK inhibitor was measured using the colorimetric XTT assay. A549 and HPFcells were seeded in a 96-well plates (5x10^3/well) and cultured for 24 h in 100 µl of complete medium.. After adhesion, cells were treated with the selected PERK inhibitory compound in a wide concentration range (3 µM, 6 µM, 12 µM, 25 µM, 50 µM, 75 µM, 100 µM, 50 mM) or 0.1% DMSO, which was used as a solvent for the investigated compound, and incubated for 16, 24 and 48h, respectively. Untreated cells cultured in a complete medium and incubated for 16, 24 or 48h, served as negative control, whereas the positive control constituted cells incubated with 100% DMSO. Absorbance was measured at wavelengths of 450 nm using the Synergy HT spectrophotometer.

The alkaline version of comet assay (ACA) was used to study the genotoxic impact of the investigated PERK inhibitor on A549 and HPF cells. A549 and HPF cells were seeded at 2 × 10^5 in 6-well plates in 2 mL of complete medium. Subsequently, A549 and HPF cells were exposed to tested compound at a wide range of concentrations (3-100 µM) or the solvent, 0.1% DMSO and incubated for 24h. Cells suspended in 5% DMSO were used as a positive control, whereas cells cultured only in complete medium, constituted a negative control. Furthermore, the effect of the PERK inhibitor was analyzed in Th-treated A549 cells. A549 cells were seeded in 6-well plates at 2 × 10^5 in 2 mL of complete medium and cultured for 24 hours. Next, the A549 cells were pre-incubated for one hour with 2 mL of complete medium containing tested compound at the concentrations of 3 µM and 50 µM. Following this, A549 cells were treated with 50 nM Th. Some cells were treated only with Th at 50 nM. The positive control constituted A549 cells treated with 5% DMSO, whereas A549 cells incubated in 2 mL of complete medium were used as the negative control. Following the treatment, all the samples were incubated for 24 hours. The cells were suspended in 0.37% low melting point (LMP) agarose placed on microscope slides pre-coated with normal melting point (NMP) agarose. The preparations were incubated in pH 10 lysis buffer and TritonX-100 at a final concentration of 1% for one hour at 4°C. Next, the preparations were incubated in development buffer for 20 min at 4°C, and then the samples were submitted to electrophoresis in the electrophoretic buffer at 4°C. The preparations were rinsed with distilled water three times and left to dry at RT. Then, the preparations were stained with a fluorescent dye (DAPI) and analyzed under a fluorescence microscope. The DNA damage in cells was measured by the percentage of DNA content in the comet tail.

The caspase-3 activity in A549 and HPF cells was analyzed using the Caspase-3 Assay Kit. A549 and HPF cells were seeded in 6-well plates (5 × 10^5/well) and cultured for 24 hours in a complete medium. Next, A549 and HPF were treated with the tested PERK inhibitor at a concentration of 3 to 100 µM or with the solvent 0.1% DMSO for 24 hours. Cell incubated for 16h with 1 µM of staurosporine were used as a positive control, whereas cells incubated for 24 hours complete medium constituted a negative control. Moreover, to investigate the effect of investigated compound on Th-treated A549 cells, the cells were seeded in 6-well plates (5 × 10^5/well) and cultured for 24 hours in complete medium. Next, A549 cells were preincubated for one hour with the complete medium containing tested PERK inhibitor at 3 µM and 50 µM concentrations, before being exposed to 500 nM Th for 24 hours. Some A549 cells were treated only with Th (500 nM, 24 h). The positive controls consisted of cells treated with 1 µM staurosporine for 16 hours, and the negative controls of cells cultured for 24 hours alone in complete A549 growth medium. The protein concentration was measured by a standard Bradford assay, with BSA used as a protein standard. For each assay, the cell lysate containing 100 µg protein was used. 2 X Reaction Buffer was then added to each sample, and the samples were incubated for two hours at 37°C. After the incubation, the p-NA absorbance was detected at 405 nm wavelength using the Synergy HT spectrophotometer.

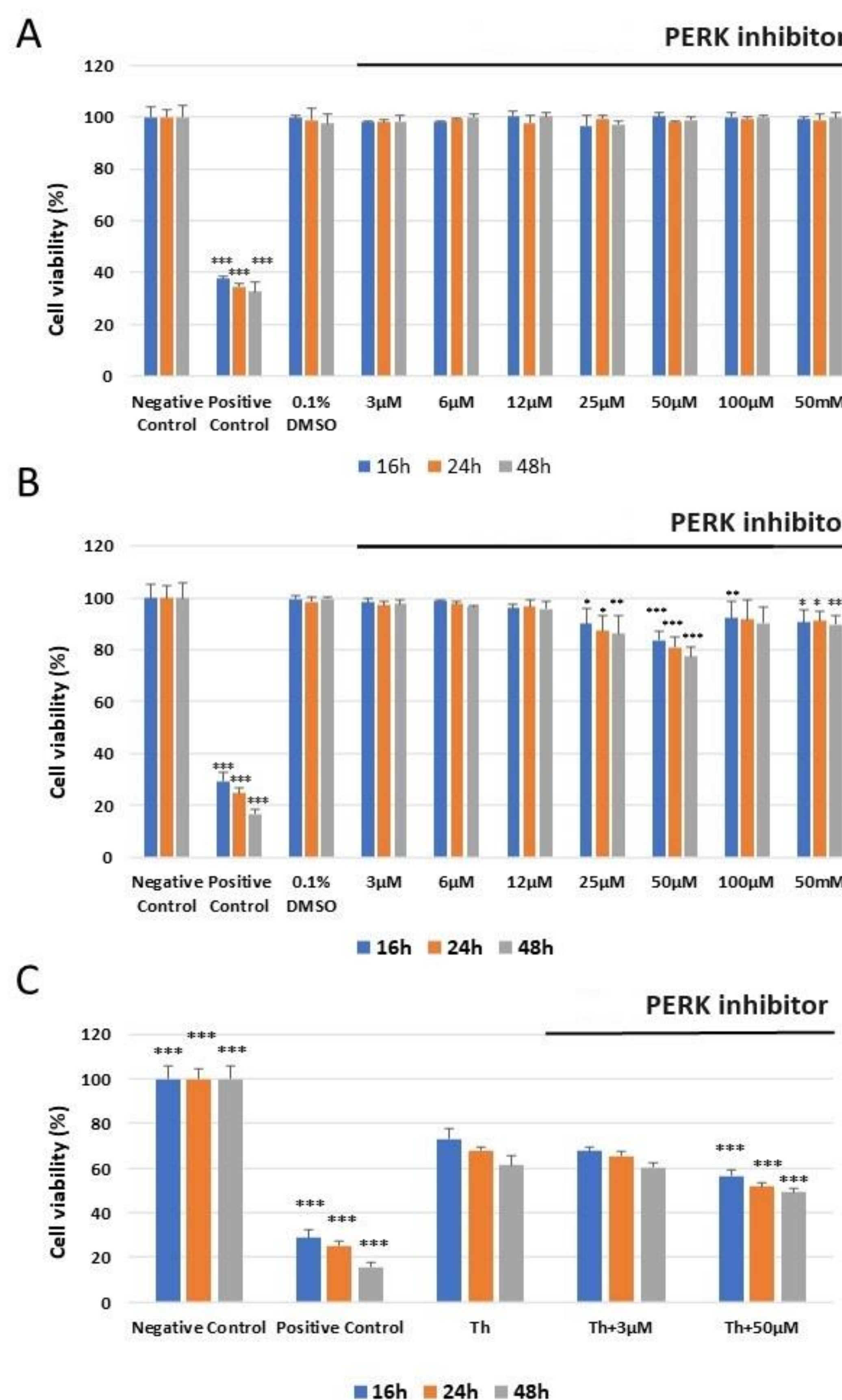

Figure 2. Analysis of the cellular toxicity of the PERK inhibitor toward HPF cells (A), A540 cells (B) and viability of A549 cells treated with Th alone and ER-stressed A549 cells upon treatment with the PERK inhibitor (C) performed by the XTT assay. All experiments were performed in triplicate, values are expressed as mean ± SEM, n = 3. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the negative control (A, B) and Th (C). Negative Control - untreated HPF (A) and A549 (B, C) cells; Positive Control - HPF (A) and A549 (B, C) cells treated with 99.9% dimethyl sulfoxide; 0.1% DMSO - HPF (A) and A549 (B) cells treated with the solvent, 0.1% dimethyl sulfoxide; Th - thapsigargin-treated A549 cells (ER-stressed A549 cells).

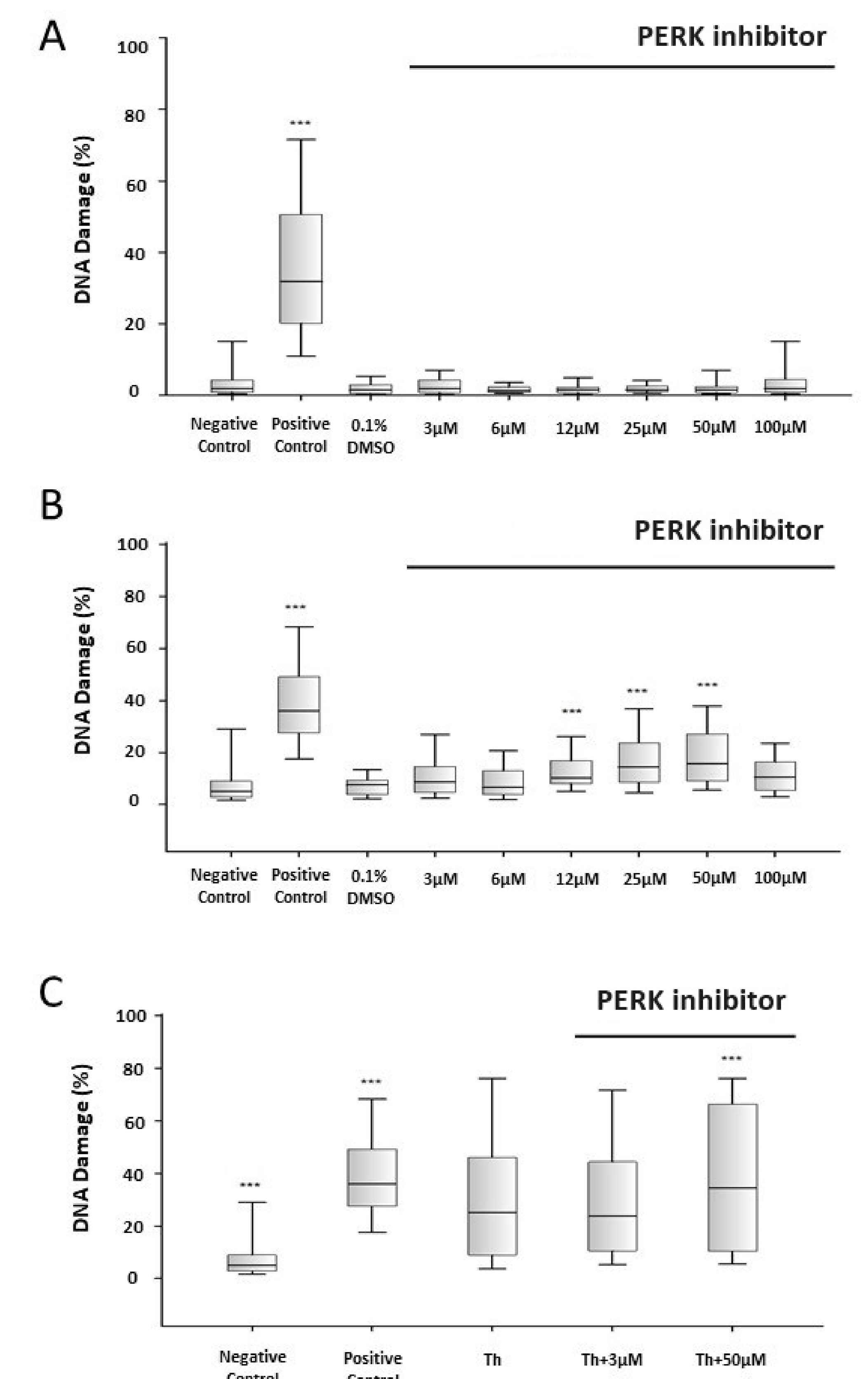

Figure 3. Analysis of genotoxicity in HPF (A) and A549 (B) cells treated with the PERK inhibitor and in ER-stressed A549 cells treated with tested compound (C), performed by the comet assay. All experiments were performed in triplicate. The value of cells scored for individual experiment was 100. Box plots show median, first and third quartiles, minimum and maximum values. *** $p < 0.001$ versus the negative control (A, B) and versus Th (C). Negative Control - untreated HPF (A) and A549 (B, C) cells; Positive Control – HPF (A) and A549 (B, C) cells treated with 99.9% dimethyl sulfoxide; 0.1% DMSO – HPF (A) and A549 (B) cells treated with the solvent, 0.1% dimethyl sulfoxide; Th - thapsigargin-treated A549 (ER-stressed A549).

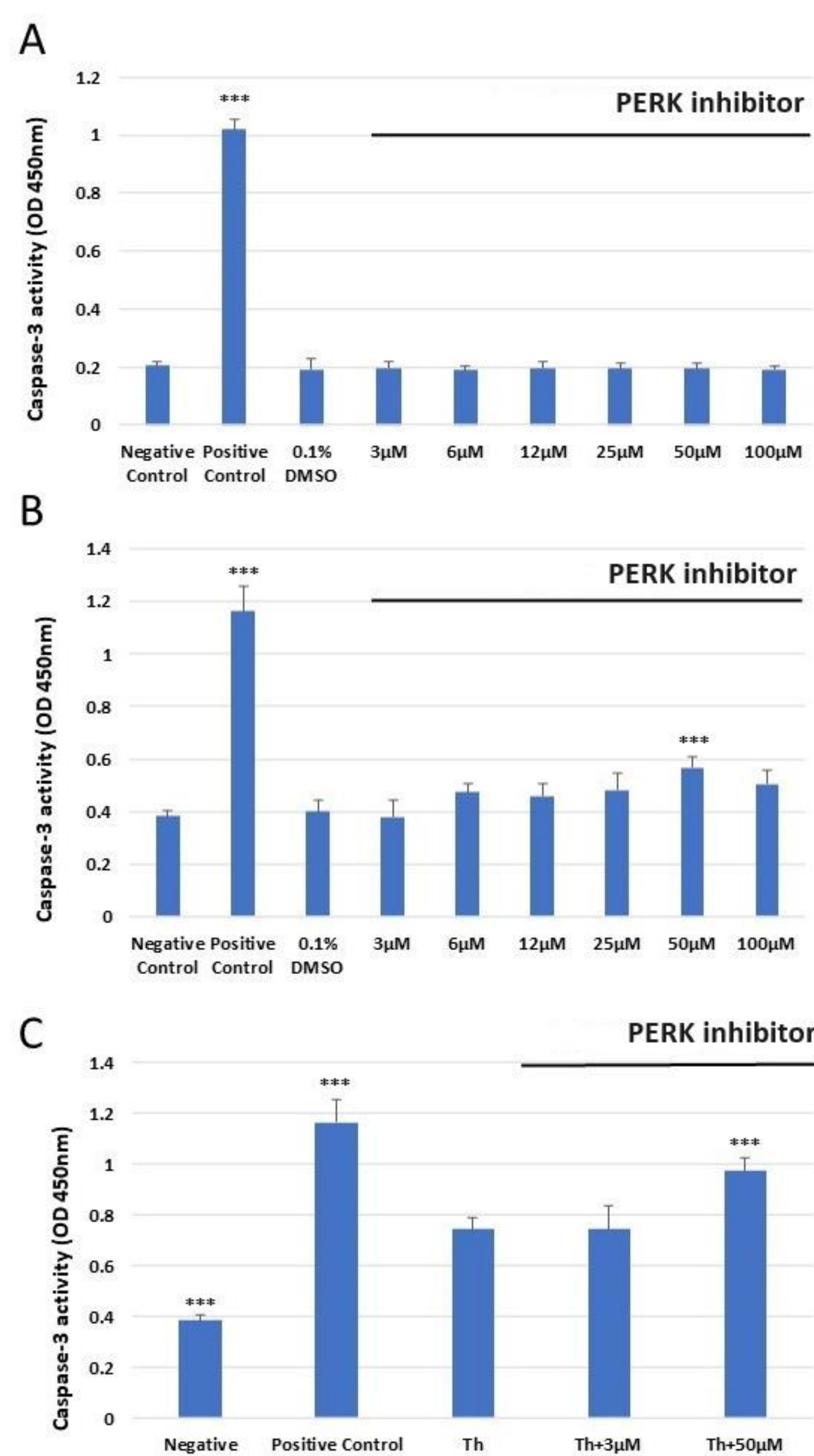

Figure 4. Evaluation of the level of apoptosis in HPF (A) and A549 (B) cells or ER-stressed A549 cells (C) after exposure to PERK inhibitor. Results determined by caspase-3 assay. All experiments were performed in triplicate, values are expressed as mean ± SEM, n = 3. *** $p < 0.001$ versus the negative control (A, B) and versus Th (C). Negative Control - untreated HPF (A) and A549 (B, C) cells; Positive Control – HPF (A) and A549 (B, C) cells treated with 1 µM staurosporine; 0.1% DMSO – HPF (A) and A549 (B) cells treated with the solvent, 0.1% dimethyl sulfoxide; Th - thapsigargin-treated A549 cells (ER-stressed A549).

Results and conclusions:

No significant cytotoxicity was noted toward HPF cell line at any applied concentrations of the tested PERK inhibitor or 0.1% DMSO after 16, 24 or 48 hours in comparison with the negative control. However, obtained results demonstrated a significant decrease in the percentage of viable A549 cells following the 16, 24 or 48-hour incubation with 50 µM of tested compound in comparison with the negative control. Also, 0.1% DMSO did not evoke significant cytotoxicity in A549 cells following the 16, 24 or 48-hour incubation as compared to the negative control. Moreover, the results showed significant decrease in viability of A549 cells with Th-induced ER stress conditions after their treatment with 50 µM of investigated compound in comparison with untreated ER-stressed A549 cells at all incubation Times – figure 2.

0.1% DMSO and the investigated PERK inhibitor did not induce a significant DNA damage in HPF cells at any concentration after 24-hour incubation in comparison with the negative control. In contrast to HPF cells, the highest increase in the DNA damage was observed in A549 cells treated with 50 µM of tested inhibitor as compared to the negative control. 0.1% DMSO did not evoke DNA damage in A549 cells. Additionally, obtained results demonstrated a significant increase in the DNA damage in the ER-stressed A549 cells treated with 50 µM of investigated compound in comparison with the untreated ER-stressed A549 cells – figure 3.

We noted a significant increase in the caspase-3 activity both in HPF and A549 cells after their treatment with 1 µM staurosporine for 16 hours. No significant increase in caspase-3-dependent apoptosis was observed after 24-hour exposure of HPF to investigated compound (3–50 µM). Furthermore, we demonstrated a relevant increase in caspase-3 activity in A549 cells treated with 50 µM of investigated compound as compared to the negative control. Inhibitor solvent 0.1% DMSO did not induce an increase in caspase-3 activity both in HPF and A549 cell lines. Additionally, obtained results demonstrated a significant increase in the ER-stressed A549 cells treated with 50 µM of tested compound in comparison with the untreated ER-stressed A549 cells – figure 4.

Thus, the continuation of this research work may prove vital to the development of novel, specific anticancer treatment strategy. Further research should be conducted to gather detailed knowledge about the PERK-dependent UPR signaling pathway mechanisms in cancer cells.