International Doctoral School
Medical University of Lodz

MEDICAL UNIVERSITY OF LODZ

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

PhD candidate: lek. dent. Adam Wawrzynkiewicz,
Auxiliary promoter: dr n. med. Wioletta Rozpędek-Kamińska,
Promoter: prof. dr hab. n. med. Ireneusz Majsterek
Department of Clinical Chemistry and Biochemistry, Medical University of Lodz

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 colorectal cancer.

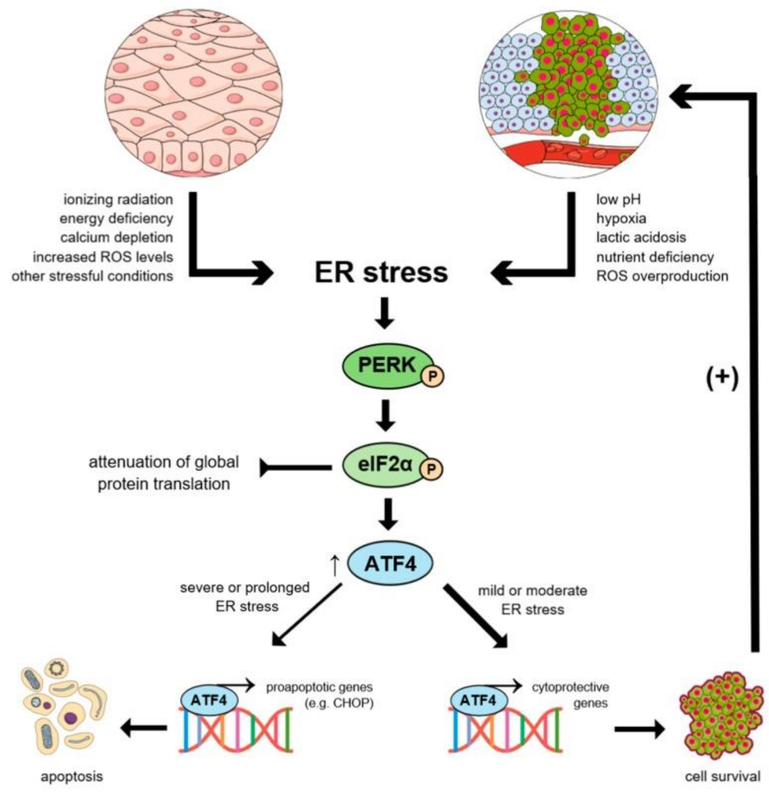
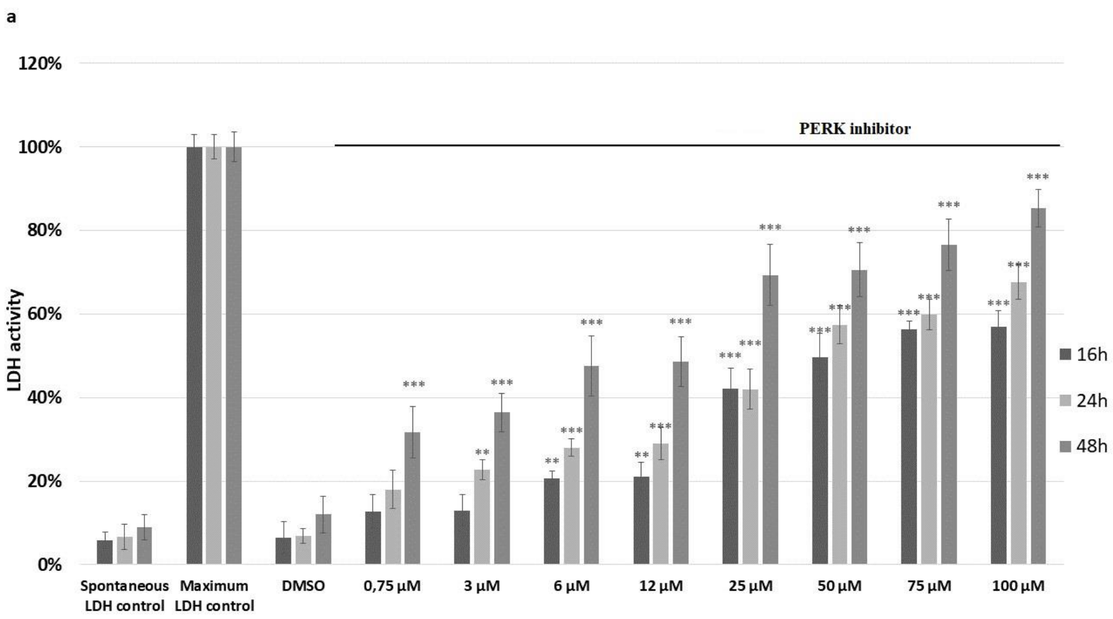


Figure 1. Unfolded Protein Response (UPR)



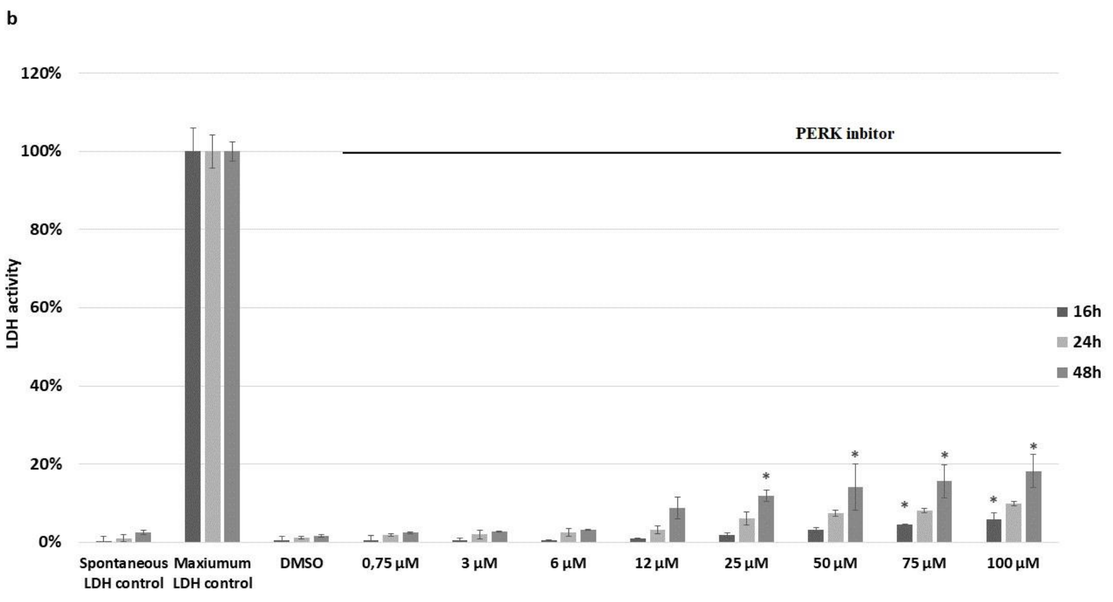


Figure 2. Evaluation of the cytotoxicity effect of small-molecule PERK inhibitor on HT-29 and CCD 841 CoN cell lines via the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit. All values were compared to the spontaneous LDH control. Bar diagrams show the percentage of LDH activity after treatment of HT-29 (a) and CCD 841 CoN (b) with the tested PERK inhibitor at a concentration range from 0.75 µM to 100 µM or 0.1% DMSO and 16, 24 or 48h incubation, respectively. Data are expressed as mean ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 versus spontaneous LDH control.

Materials and methods:

The anti-tumor properties of previously selected small-molecule PERK inhibitor were examined on commercially available human colon adenocarcinoma cell line HT-29 and the normal human colon epithelial cell line CCD 841 CoN.

The cytotoxicity of the selected PERK inhibitor was measured using the The lactate dehydrogenase (LDH) Cytotoxicity Assay Kit. All of the experiments were performed in triplicate with similar results. HT-29 and CCD 841 CoN cells were seeded in a 96-well plates ($8x10^3$/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 (0.75 µM, 3 µM, 6 µM, 12 µM, 25 µM, 50 µM, 75 µM, 100 µM) 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 a spontaneous LDH control. Absorbance was measured at wavelengths of 490 nm and 680 nm (background) using the Synergy HT spectrophotometer.

Apoptotic cell death was measured by flow cytometry using the FITC Annexin V Apoptosis Detection Kit. Above-mentioned analysis is based on FITC-conjugated Annexin V (Annexin V-FITC) that binds to phosphatidylserine at the cell surface of the apoptotic cells and the propidium iodide (PI) that constitutes a marker of cell membrane permeability. HT-29 and CCD 841 CoN cells were double stained with annexin V, as a marker of early apoptosis, and PI as a marker of cell membrane disintegration, necrosis and late apoptosis. The cultured cells were treated with the concentrations 6 µM and 50 µM of small-molecule PERK inhibitory compound and incubated for 24h. Cells treated with staurosporine at concentration of 1 µM for 16 h constituted a positive control, whereas 0.1% DMSO-treated cells and incubated for 24h served as a negative control. Cell suspensions were analysed by flow cytometry using the Beckman Coulter CytoFLEX. The obtained data were analyzed using the Kaluza analysis 1.5A software.

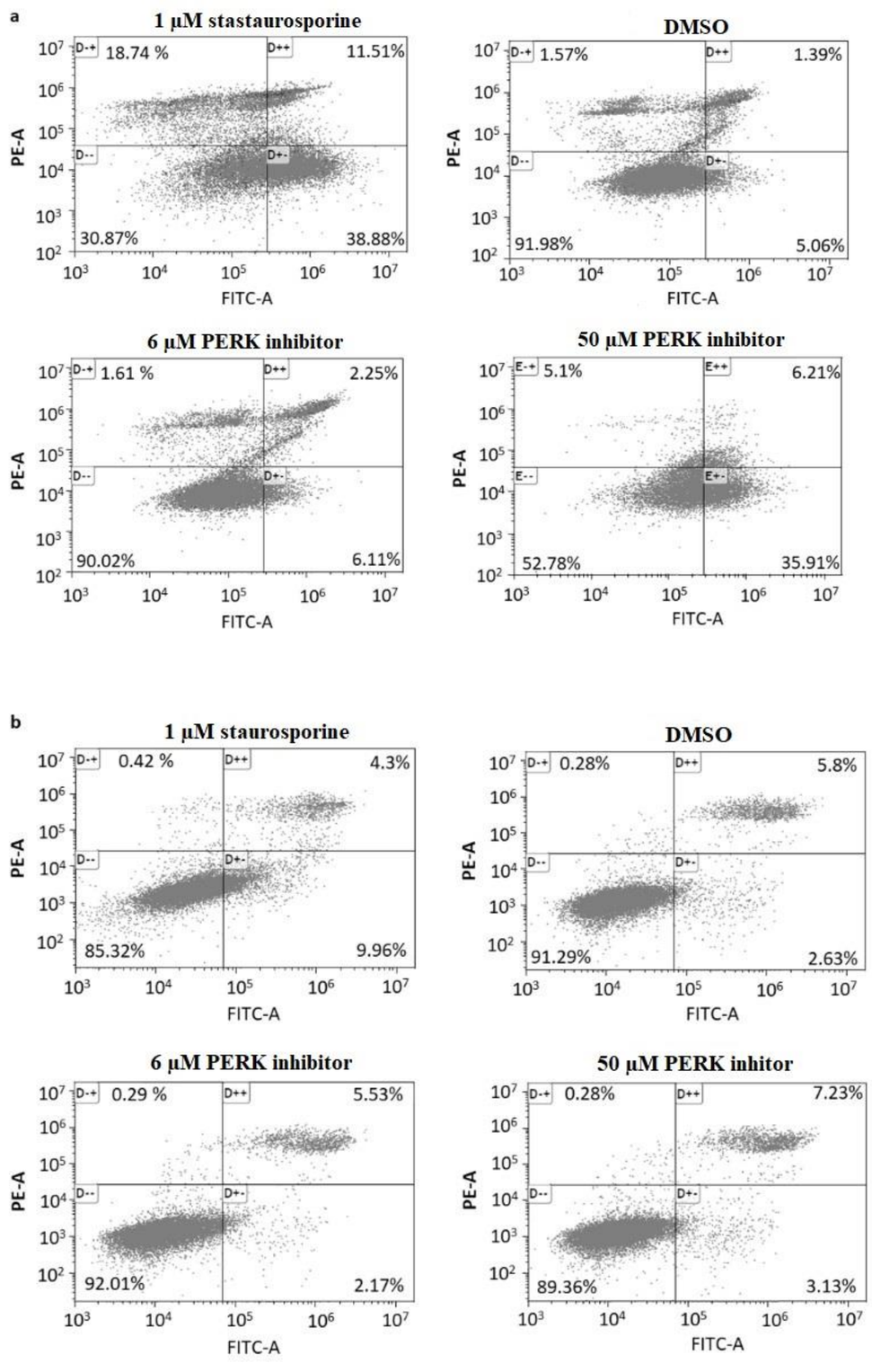


Figure 3. Flow cytometric FITC annexin V/PI double staining analysis of apoptosis. HT-29 and CCD 841 CoN cells were treated with 6 µM and 50 µM of small-molecule PERK inhibitor for 24h. 1 µM xin V negative, PI positive) cells for HT-29 (a) and CCD 841 CoN (b) cells. staurosporine-treated cells served as a positive control, whereas cells incubated with 0.1% DMSO constituted a negative control. Dot plot graphs indicate the percentage of viable (FITC annexin V negative, PI negative), early apoptotic (FITC annexin V positive, PI negative) late apoptotic (FITC annexin V positive, PI positive) and necrotic (FITC anne

Results and conclusions:

Analysis of cytotoxicity, measured via the colorimetric LDH assay demonstrated that investigated small-molecule PERK inhibitor significantly inhibited HT-29 cells viability in a dose- and time-dependent manner. In comparison with HT-29 cells, no significant cytotoxic effect was observed in non-cancerous CCD 841 CoN cells at any concentration of selected compound and time of incubation used. As compared to controls, a significant cytotoxic effect of tested PERK inhibitor toward HT-29 cells was noticed at a concentration of 50 µM at all incubation times. A similar response was observed after treatment of HT-29 cells with tested compound at higher concentrations (75 µM and 100 µM). Based on the obtained data, in further experiments cells were incubated with the selected small-molecule PERK inhibitor at 6 µM and 50 µM – figure 2.

Activation of apoptosis was analysed with FITC annexin V/PI double staining by flow cytometry in HT-29 and CCD 841 CoN cell lines exposed to 6 µM and 50 µM of small-molecule PERK inhibitor for 24h. After treatment with 1 µM staurosporine for 16h, a significant number of cells of both investigated cell lines undergo apoptosis as compared to control cells, incubated for 24 with the 0.1% DMSO. Interestingly, the results demonstrated that approximately 42% of HT-29 cells treated with 50 µM of selected PERK inhibitor were at the early and late stages of apoptosis. Moreover, treatment with 50 µM of tested compound demonstrated no significant increase in HT-29 necrotic cells. Obtained results did not indicate a significant number of HT-29 dead cells treated with small-molecule PERK inhibitor at a concentration of 6 µM (8.36% of cells both at the early and late stages of apoptosis. However, after 24h treatment with tested compound at a concentration of 50 µM only 10% of CCD 841 CoN cells were in early and late apoptosis. There was no significant number of dead CCD 841 CoN cells after their 24h treatment with small-molecule PERK inhibitor at a concentration of 6 µM (7.7% of cells both at the early and late stages of apoptosis). In addition, there was no increase in necrotic cells after 24h treatment of CCD 841 CoN with tested compound both at 6 µM and 50 µM concentrations when compared to control cells incubated for 24 with the 0.1% DMSO – figure 3.

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.