Inhibition of PERK-dependent pro-adaptive signaling pathway as a promising approach for cancer treatment

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ABSTRACT: Endoplasmic Reticulum (ER) is an organelle that is vital for cell growth and maintenance of homeostasis. Recent studies have reported that numerous human diseases, including cancer, are strictly connected to disruption of ER homeostasis. In order to counteract adverse intracellular conditions, cancer cells induce protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK)-dependent, pro-adaptive unfolded protein response (UPR) signaling branches. If ER stress is severe or prolonged, pro-adaptive signaling networks are insufficient, resulting in apoptotic cell death of cancer cells.

The main aim: of the study was to evaluate the biological activity of a small-molecule PERK inhibitor GSK2606414 in two cancer cell lines - human neuroblastoma (SH-SYSY) and human colorectal adenocarcinoma (HT-29) cell lines. We analyzed the level of phosphorylation of the eukaryotic initiation factor 2α (eIF2α), which is the main substrate of PERK and a subsequent activator of UPR, which under long-term ER stress may evoke apoptotic death of cancer cells.

Material and methods: In the study, we utilized commercially available cell lines of human colorectal adenocarcinoma HT-29 and human neuroblastoma SH-SYSY. Cells were exposed to the tested PERK-dependent signaling inhibitor GSK2606414 in suitable culture media with addition of thapsigargin (500 nM) to induce ER stress. To identify the protein, Western blot with specific antibodies was used. Detection of immune complexes was performed using chemiluminescence.

Results: We found a complete inhibition of phosphorylation of eIF2α and expression of GADD34 in both tested cell lines. SH-SYSY and HT-29.

Conclusions: Currently available cancer treatments are insufficient and cause various side effects. It has been assumed that utilization of small-molecule inhibitors of the PERK-dependent signaling pathway, like GSK2606414, may switch the pro-adaptive branch of UPR to its pro-apoptotic branch. It is believed that the tested inhibitor GSK2606414 may become a promising treatment for many cancer types.

KEYWORDS: cancer, PERK, eIF2α, PERK inhibitor, GSK2606414, apoptosis

INTRODUCTION

The endoplasmic reticulum (ER) is an organelle which is mainly responsible for proper protein folding, biosynthesis of phospholipids, and maintenance of calcium and redox homeostasis. ER is an interconnected cellular membrane network that plays a vital role in cell growth and homeostasis. There is ample evidence that many human diseases, including cancer, type 2 diabetes mellitus, metabolic, cardiovascular, and neurodegenerative diseases are strictly linked to disruption of ER homeostasis, which may be caused by various pathological conditions. Cancer cells are characterized by an uncontrolled proliferation, insufficient formation of new blood vessels, and pathological architecture. The above-mentioned features are the main cause of low oxygen level in blood, i.e., hypoxia. Hypoxia directly evokes ER stress through accumulation of misfolded or unfolded proteins within the ER lumen. In response to pathological conditions, the unfolded protein response (UPR) signaling pathway is activated. Interestingly, UPR plays a dual role; it may be pro-adaptive or pro-apoptotic depending on the severity of stress and time of cell exposure to unfavorable conditions. The primary role of the UPR signaling networks is to protect cancer cells from apoptotic cell death. However, when ER stress is severe and prolonged, cells may undergo apoptosis.

Aggregation of aberrant proteins within the ER lumen evokes activation of UPR, which consists of three branches activated via three specific transmembrane receptors - inositol regulating enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), which is strictly linked to cancer development and progression. Under ER stress, PERK undergoes oligomerization and trans-autophosphorylation. Activated PERK has a significant impact on protein translation, since it triggers phosphorylation of eukaryotic initiation factor 2α (eIF2α) at the Ser51 subunit α. This, in turn, results in attenuation of global protein synthesis with preferential translation of selected mRNAs such as ATF4. Moreover, activated PERK evokes phosphorylation of nuclear factor-erythroid 2-related factor-2 (Nrf2) and DNA damage-inducible protein (GADD34) that activate signaling pathways leading to apoptotic cell death, which is a prominent mechanism for maintaining homeostasis via elimination of abnormal cells such as cancer cells.

The purpose of this work was to assess the biological activity of a small-molecule PERK inhibitor GSK2606414 in cancer cell lines by analyzing phosphorylation of eIF2α, which is an activator of pro-apoptotic UPR signaling pathways.
MATERIAL AND METHODS

In the study, we utilized commercially available cell lines of human colorectal adenocarcinoma HT-29 (ATCC® HTB-38™) and human neuroblastoma SH-SY5Y (ATCC® CRL-2266™). Cell culture was conducted according to the manufacturer’s instructions using the ATCC-McCoy’s medium for HT-29 and the ATCC-Eagle’s Minimum Essential Medium (EMEM) and F-12K Medium (Kaighn’s Modification of Ham’s F-12 Medium) at a 1:1 ratio for SH-SY5Y. In addition, 10% fetal bovine serum (FBS - ATCC 30-2020™), penicillin (100 U/ml), and streptomycin (100 mg/ml) were added to the media. Cells were incubated at 37 °C in a humidified atmosphere (95% air and 5% CO₂).

Cells were exposed to the tested PERK-dependent signaling inhibitor GSK2606414 (at a concentration of 1 μM; GlaxoSmithKline) in suitable culture media with addition of thapsigargin (500 nM) to induce ER stress (Sigma Aldrich) with incubation time of 3h. In parallel, studies were carried out with thapsigargin and without the PERK inhibitor GSK2606414 as positive controls (incubation time of 3 h). Additionally, cell cultures that were not treated with GSK2606414 inhibitor or thapsigargin served as negative controls.

METHODS

Western blot. Cells were harvested and lysed using a protein extraction reagent (Pierce, Rockford, IL, USA). Then, protein concentration was determined using a protein kit (Pierce, Rockford, IL, USA) using bicyclic acid (BCA). Electrophoresis of samples containing 80 μg of total protein was performed in a 12% polyacrylamide gel in a vertical electrophoresis machine (Bio-Rad, Richmond, CA, USA). After electrophoresis, the proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) during the wet electrotransfer. Then, the membrane was blocked with 5% skimmed milk in TBS / 0.1% Tween for 1 hour to prevent non-specific binding of proteins to the membrane. In the next step, the membrane was incubated for 16h at 4°C with specific primary antibodies (1: 1000; Cell Signaling Technologies), and then for 1h with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies 1: 5000). Detection of immune complexes was carried out using the Fisher Scientific EMD Millipore Immobilon™ Western Chemiluminescent HRP Substrate (ECL chemiluminescence system). The following antibodies were used: p-eIF2α, eIF2α, β-actin (Cell Signaling Technologies).

RESULTS

Figure 1 shows the results of immunodetection of p-eIF2α and eIF2α in SH-SY5Y cell lines. Figure 2 shows the results of immunodetection of p-eIF2α and eIF2α in HT-29 cell lines. β-actin was used as a loading control for the analyzed samples. In both cell lines, ER stress was not observed in cells that were not treated with the GSK2606414 inhibitor and thapsigargin (positive control). ER stress was observed in SH-SY5Y and HT-29 cell lines as evidenced by an increased expression of p-eIF2α proteins after 3h incubation with thapsigargin (negative control, Kₜ). Complete inhibition of phosphorylation of eIF2α was observed in SH-SY5Y and HT-29 cell lines that were simultaneously treated with GSK2606414 and thapsigargin and then incubated for 3h.

DISCUSSION

Recent studies have reported that causes of numerous human diseases, such as cancer, neurodegenerative diseases, type 2 diabetes mellitus, atherosclerosis, and metabolic diseases can be found on a molecular level [1]. After cardiovascular diseases, cancer is the second most common cause of death in highly developed countries [2]. It has been reported that rapid tumor growth, invasion, and angiogenesis are strictly associated with an activation of the PERK-dependent signaling networks and subsequent phosphorylation of eIF2α. Thus, at an early stage of cancerogenesis, the UPR network is activated in cancer cells, which has not been observed in healthy cells that surround cancer cells with excessive, uncontrolled proliferation [3, 4]. Therefore, ER stress, induced by hypoxia within cancer cells, results in upregulation of eIF2α phosphorylation [5]. Research indicates that long-term ER stress may induce apoptosis of cancer cells [6]. Therefore, upon severe or prolonged ER stress the pro-apoptotic branch of the UPR response is insufficient to maintain homeostasis. Subsequently, the pro-apoptotic UPR branch is activated, which leads to cancer.
cells death [7]. Currently, molecular mechanisms responsible for the switch from the pro-adaptive to the pro-apoptotic PERK-dependent signaling pathway remains unclear [8].

Unfortunately, currently available cancer treatments are insufficient and cause numerous side effects [9, 10]. A large body of evidence indicates that inhibition of the UPR signaling network may be an effective treatment against various human diseases including cancer. Utilization of small-molecule inhibitors may trigger a molecular switch of the PERK-dependent signaling branches within cancer cells from the pro-adaptive to the pro-apoptotic branch [11]. It has been demonstrated that GSK2606414 significantly inhibits PERK in vitro in cell lines and in vivo in the mouse model of human pancreatic tumor xenografts [12]. Moreover, Moreno et al. have showed that GSK2606414 may inhibit neurodegeneration in prion-infected mice. GSK2606414 triggered inhibition of the PERK-mediated signaling network, resulting in a significant decrease in PERK and eIF2α phosphorylation [13]. Moreover, a combined use of GSK2606414 and proteasome inhibitors such as Velcade might be associated with a significantly less toxic therapy against multiple myeloma [14, 15]. In our study, we have shown that the GSK2606414 inhibitor can significantly inhibit the PERK-dependent signaling pathway in two cancer cell lines, namely, SH-SY5Y and HT-29. Further experiments, both in vitro experiments with different cancer cell lines and in vivo experiments in animal models of human diseases, are indicated. Comprehensive knowledge of GSK2606414 activity may help develop other compounds with similar properties but with lower cytotoxicity. The above-mentioned new generation anti-cancer drugs may be used as novel treatments that would induce apoptotic death of cancer cells.

REFERENCES
