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Readthrough Intervention Increases ER Stress in Wolfram Syndrome

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Abstract

Aim: Wolfram syndrome (WFS) is an example of inherited endocrine and neurodegenerative disease due to increased ER stress with no causal treatment. WFS is an autosomal recessive syndrome caused by biallelic mutations in *WFS1* gene. Some of these mutations result in premature termination codons (PTCs). Some prospects for the causal treatment of WFS patients could give a PTCs readthrough intervention. The use of ataluren (formerly PTC124) can result in bypassing the PTCs and lead to a continuation of translation. The aim of the study was to evaluate the repairing potential of ataluren in a cell model of WFS caused by PTCs.

Materials and methods: Diagnosis of WFS was confirmed by Sanger sequencing of the *WFS1* gene. ER stress induction (Tunicamycin; Sigma-Aldrich, Germany) with subsequently using PTC124 (Ataluren, Selleckchem, USA) were performed on fibroblasts obtained from skin biopsies of WFS patients and healthy individuals. The evaluation of ER stress induction was conducted by analysis of mRNA expression of recognized markers of the ER stress (7900HT Real Time PCR; Applied Biosystems, USA).

Results: Expression of specific markers of ER stress in patients with WFS was increased after using tunicamycin, with the highest value after 8 hours of the ER stress induction. The highest increase in mRNA expression after application of PTC124 in combination with DMSO in relation to DMSO itself was observed for GRP78 (p=0.0013). Fold change was 3.41 ± 0.73.

Conclusion: It seems that PTC124 by the ER stress increasing cannot be used as a potential causal treatment for the WFS patients.

Keywords: Wolfram syndrome; PTC124; Treatment; ER stress

Introduction

Wolfram syndrome (WFS) is an example of a rare genetic syndrome resulting from biallelic mutation of *WFS1* gene. The prevalence of WFS in the European population is very low (about 1/500,000 to 1/770,000). In all of the WFS patients diabetes mellitus together with optic nerve atrophy are observed. No causal treatment is currently available in this syndrome [1,2].

WFS is caused by recessive mutations in the *WFS1* gene localized on chromosome 4p16.1 [3]. Wolframin – as a product of *WFS1* gene – is an integral component of the endoplasmic reticulum and is expressed in many tissues and organs (e.g., brain, heart, pancreas, liver, muscles). This wide tissue distribution of wolframin contributes to the pleiotropic effects of mutations of the *WFS1* gene. Wolframin is in fact a kind of "gatekeeper" that protects cells from ER (endoplasmic reticulum) stress which occurs due to accumulation of endogenous protein synthesis products [4]. Thus, the loss of its function in particular cells implies an increased ER stress resulting in apoptosis of the affected cells and many neurodegenerative and endocrine symptoms. Currently, above 200 mutations resulting in WFS have been described [5]. Some of them are the mutations of premature termination codons (PTCs) [6].

It seems that some prospects for the causal treatment of patients with WFS can give a recently discovered phenomenon – a readthrough of PTCs. The use of a chemical compound that bypasses premature stop codons (stop codons are reading as glycine) results in a continuation of translation. Chemical compounds with the above described properties are e.g. aminoglycosides, including gentamicin. However, they have numerous side effects [7,8]. Ataluren (formerly PTC124), another readthrough-promoting compound, is less toxic and was approved to treat patients with Duchenne muscular dystrophy who have a nonsense mutation in the dystrophin gene [9].

In the current study, we conducted *in vitro* studies on the phenomenon of ER stress in WFS patients with confirmed mutations of premature stop codons and the possibilities of repairing a genetic defect by using ataluren.

Materials and Methods

The study protocol was approved by the University Bioethics Committee at the Medical University in Lodz, Poland (RNN/133/10/KE). Patients and/or their parents gave written informed consent for participation in the study. Diagnosis of WFS was confirmed by direct sequencing of the *WFS1* gene and/or multiplex ligation-dependent probe amplification (MLPA; SALSA MLPA P163 GJB-WFS1 probemix, MRC-Holland, The Netherlands), as described previously [10]. Skin biopsies in WFS patients (n=2) and healthy volunteers (n=2) were performed.

Cell cultures

Fibroblasts obtained from skin biopsies were grown in DMEM medium (Sigma-Aldrich, Germany) supplemented with 10% FBS

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Heat Inactivated (Invitrogen, Germany) and a solution of penicillin and streptomycin (Sigma-Aldrich, Germany). Cells were cultured in polystyrene bottles at 37°C in a humidified atmosphere with 5% CO2 concentration. After reaching confluence, the fibroblasts were passaged with a solution containing 0.25% trypsin and EDTA (Invitrogen, Germany). Then a solution of trypsin was added to the cells washed with PBS solution without calcium and magnesium (PAA Laboratories, Canada) and they were incubated for 2-3 minutes at 37°C. Trypsinization was stopped by adding DMEM with 10% FBS. The cells were centrifuged for 5 minutes at 800 × g. The cell pellet was resuspended in a complete culture medium. Cells were passaged to the new culture bottles. The amount of cells per bottle was 0.5 x 106.

ER stress induction and cell isolation

All the cell experiments for each patient and control sample were performed in triplicates. The cell cultures were performed for 48 hours at 37°C in a humidified atmosphere with 5% CO_2 concentration with addition of PTC124 (Ataluren, Selleckchem, US) at a final concentration of 6 μg/ml. At the same time, cell cultures were conducted in a medium without PTC124 and in a medium containing DMSO (Sigma-Aldrich, Germany) at a final concentration of 0.05%. After 48 hours, the medium was changed to DMEM with 10% FBS with ER-stress inductor tunicamycin (TM, Sigma-Aldrich, Germany). After 4 and 8 hours of induction, cells were harvested using a trypsin solution (as described above). For the next part of the experiment, the cell pellet was resuspended in 1 ml of Trizol Reagent (TRI Reagent; Invitrogen, Germany). TRI Reagent was used for proper isolation and the process was conducted according to the manufactured protocol. The concentration of RNA obtained was measured using a super sensitive NanoDrop ND1000 spectrophotometer (Thermo Scientific, US).

Evaluation of ER stress induction was performed by repeatable analysis of mRNA expression of recognized markers of the ER stress: XBP1, GRP78 (HSPA5), GADD153 (CHOP) and ATF4 using the method of realtime-PCR (RT- PCR). mRNA levels after induction of ER stress and addition of PTC124 were referred to the value prior to induction.

RT-PCR experiments

Reverse transcription reactions were performed using a commercially available set of High Capacity cDNA Archive Kit (Applied Biosystems, US). cDNA was prepared from 1 mg of mRNA with random hexamer primers, according to the manufacturer`s instructions - 10 minutes 25°C, 2 hours 37°C and 4°C on a PCR thermocycler Gene (Applied Biosystems, US). The resulting cDNA was diluted to a final concentration of 5 ng/ul and constituted a matrix in further experiments.

Expression experiments

The analysis of gene expression was performed using commercially available assays. XPB1 assays: XBP1-A Hs00231936_m1 and XBP1-B Hs02856596_m1, GRP78 assay HSPA5 Hs00946084_g1, CHOP assay DDIT-3 Hs01090850_m1, ATF4 assay ATF4 Hs00909569_g1 and GAPDH (Applied Biosystems, US) which were controlled by an internal reaction while allowing a reliable determination of absolute values and the expression of XBP1, GRP78, CHOP and ATF4 genes. The analysis was carried out in the genetic analyzer 7900HT Real Time PCR (Applied Biosystems, US). Comparative analyses of each of these genes in individual patients were performed using specialized computer programs SDS2.3 and RQ 2.1 (Applied Biosystems, US). All the measurements for each time-point and sample were performed in duplicates.

Statistical analysis

Statistical analysis was performed using Statistica 12.5 (Statsoft, Poland). The quantitative values between groups were compared with the Mann-Whitney U-test. The differences between time-points were evaluated using Wilcoxon's rank test and non-parametric ANOVA [11]. A *p* value lower than 0.05 was considered statistically significant.

Results

In 10/16 (62.5%) of WFS patients, the mutations of premature stop codons were identified (Table 1).

Thus, we performed *in vitro* studies involving the induction of ER stress using ER stress inductor - TM for 4-8 h with an attempt to repair the defect by using the readthrough of PTCs intervention. For this purpose, ataluren (PTC124) was used. For the skin biopsy we have chosen the patients with homozygous p.Trp540Ter mutation in *WFS1* gene.

Based on the mRNA expression values for the markers of ER stress the effectiveness of ER stress induction on cell lines from WFS patients was confirmed. It demonstrated that the expression of all four ER stress markers was increased after the use of tunicamycin, which was most visible after 8 hours of ER stress induction (Figure 1a-d).

The results also showed that PTC124 may further increase the ER stress. The highest increase in mRNA expression after application of PTC124 was observed for GRP78 (p=0.0013). Fold change was 3.41 ± 0.73 (Figure 1a). Fold change for the increase of CHOP mRNA expression was 1.22 ± 0.32 (p=0.041) (Figure 1b), whereas fold change for the increase of ATF4 mRNA expression after 4 hours of using tunicamycin was 1.74 ± 0.24 (p=0.032) and after 8 hours - 1.22 ± 0.33 (p=0.047) (Figure 1c). A decrease in mRNA expression for XBP1 was also noted (p=0.039). Fold change was 0.58 ± 0.11 (Figure 1d).

Patient ID	Mutation (amino acid change)	Age at DM diagnosis (years)	Age at OA diagnosis (years)
WFS1	Homozygous p.Val412Ser	5	17
WFS2	p.Trp648Ter/ p.Val779Gly	3.8	5.8
WFS3	Homozygous p.Val659Ser	8	6
WFS4	Homozygous p.Ser443Arg	5	5.5
WFS5	Homozygous p.Ser443Arg	4	5
WFS6	p.Gln392Ter/ p.Tyr513Ser	7	20
WFS7	Homozygous p.Trp540Ter	4	6
WFS8	Homozygous p.Trp540Ter	5	9
WFS9	p.Ser167Glu/ p.Trp648Ter	8.7	9.7
WFS10	Homozygous p.Trp648Ter	5	9
WFS11	p.Lys190_Lys191insLys/ p.Ser236Arg	5.5	9
WFS12	**Homozygous p.Trp540Ter**	**6**	**10**
WFS13	Homozygous p.Trp540Ter	4.5	9
WFS14	p.Val142Glyfs*105/ p.Trp648Ter	5	13
WFS15	Homozygous p.Trp648Ter	8	9
WFS16	p.Arg558Cys/ p.Val412Ser	13	19

Table 1: Characteristics of the Polish patients with Wolfram syndrome.

DM – diabetes mellitus, OA – optic atrophy.

A patient with stop codon mutation whose fibroblasts were used in the experiment is indicated in bold.

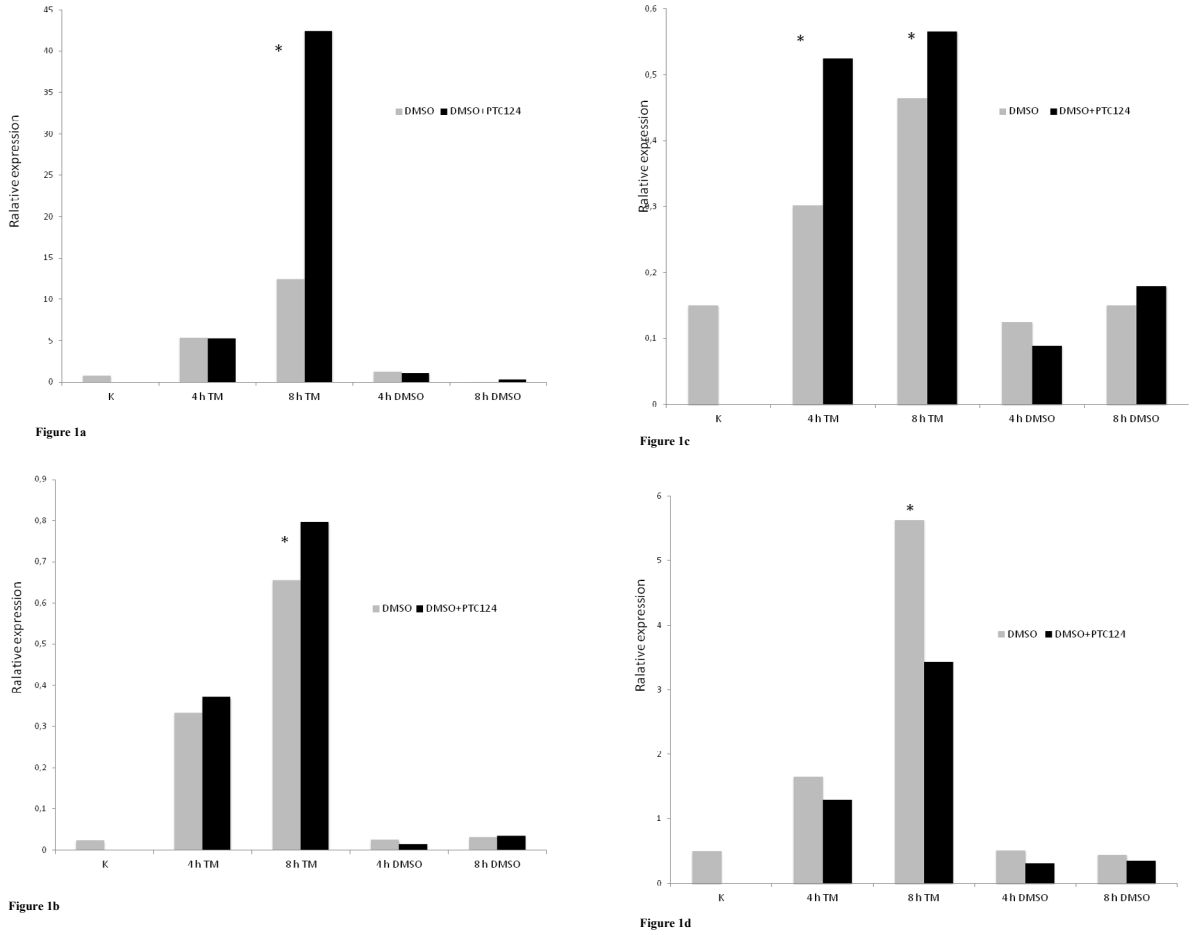

Figure 1: Changes in mRNA expression for selected ER stress markers in fibroblasts collected from the WFS patient (homozygous for p.Trp540Ter mutation) treated with tunicamycin as ER stress trigger:

* denoted p<0.05 1a. for HSPA5 (GRP78 1b. for DDIT3 (CHOP) 1c. for ATF4 1d. for ratio between splicing form and full form of XBP1.

Discussion

Wolfram syndrome is a genetic endocrine and neurodegenerative disorder which significantly reduces life-time expectancy of the patients. Currently, there is no causal therapy available and only substitutive treatment with insulin and vasopressin is given. Our approach was designed as a proof of concept study to apply a readthrough of the PTCs as an intervention in the cell model of WFS, which is consistent with the observation of nonsense mutations frequency in Polish WFS patients (62.5%).

In this study the effectiveness of ER stress induction using tunicamycin on the cell lines (fibroblasts) from patients with WFS was confirmed. The obtained values of mRNA expression for selected ER stress markers showed their increase in WFS patients after using tunicamycin. Unfortunately, PTC124 further increased the ER stress which excludes its usefulness as a potential causal treatment in the WFS patients. It was noted for GRP78, CHOP and ATF4 mRNA expression. However, mRNA expression for XBP1 after PTC124 application was decreased.

The role of the ER stress phenomenon in WFS has been already well described. So far, it is known that in WFS, in the absence of wolframin, ER stress is increased by both PERK-dependent (PKR-like ER kinase) and the activation of the transcription factor ATF6. Furthermore, an unconventional splicing of the mRNA encoding the X-box binding protein 1 (XBP1) as a result of excitation of the third pathway of ER stress by requiring inositol factor 1 (IRE1) catalyzing is observed. Activation of all three ER stress pathways lead to stimulation of specific markers of ER stress or/and proapoptotic markers, such as: ATF6, ATF4, XBP1, GRP78 (BIP) and GADD153 (CHOP). As a result of increased ER stress, ultimate cell death is observed and as a consequence - the appearance of specific clinical symptoms in patients with WFS is noted [4,12]. Moreover, it is known that CHOP should be treated as the most proapoptotic marker, whereas GRP78 is rather a marker of protein accumulation in the ER and increased XBP1 expression indicates that the ER homeostasis has been preserved [13-16].

In our study, increased results of mRNA expression for GRP78, CHOP, ATF4 with reduced mRNA expression for XBP1 may mainly show the disturbances of the ER homeostasis and activation of protein accumulation with some degree of apoptosis induction.

At the same time, it is known that nonsense mutations in the *WFS1* gene lead to PTCs in some WFS patients, resulting in the absence of biologically active and stable wolframin [6,17].

Currently, there is no possibility to repair this defect in WFS patients. However, it seems that a return of function of wolframin could be achieved through suppression of the premature stop codons. It involves binding a chemical compound to a smaller ribosomal subunit and uncoupling the accuracy of the codon-anticodon system. It can result in a continuation of translation and synthesis of a biologically active protein due to an incorporation of an amino acid at the codon stop site [18]. The chemical compounds with the above described properties can be, for example, aminoglycoside antibiotics, but their supply leads to potential side-effects in the patients [7,8]. In order to improve their efficiency, new antibiotics were found and used in the "readthrough of PTCs" phenomenon [19-21].

Another compound with properties of bypassing the premature stop codons is PTC124, which can be used orally and does not cause serious side effects [9]. Its major advantage is an absence of influence on normal stop codons which is associated with the lack of interfering with the structure of other proteins [22,23]. The phenomenon of PTCs readthrough using ataluren has successfully passed a phase of *in vitro* tests and animal studies in many diseases including: cystic fibrosis, lipofuscinosis, mucopolysaccharidosis VI and Duchenne muscular dystrophy [24-28]. PTC 124 was also tested in the treatment of such diseases as: Usher syndrome [29], lysosomal diseases [30] and genetic eye disorders [31].

So far, clinical trials for selected genetic diseases related with the presence of mutations with premature stop codons such as cystic fibrosis have been conducted [32-34]. Moreover, several non-acidic ataluren analogues were selected and tested in cell lines carrying nonsense mutations also in the CFTR gene [35].

Thus, we tried to verify whether the *in vitro* studies using ataluren can become the starting point for the use of the readthrough of PTCs intervention in future clinical trials aimed at the causal treatment of patients with WFS. Unfortunately, the results obtained in the study confirmed that ataluren may exacerbate the ER stress. It seems possible taking into consideration the mechanism of its action including the insertion of an amino acid at the premature stop codon site, followed by the synthesis of an active protein. Despite the potential positive effect of ataluren, it may, however, result in the accumulation of protein synthesis products and thereby lead to the increase of ER stress. A limitation of this study is the lack of results regarding the amount of obtained protein.

Conclusion

For the first time the *in vitro* studies using ataluren as a potential causal treatment in WFS patients were performed. The experimental studies on skin fibroblasts obtained from WFS patients allowed to create an effective model of ER stress induction in the patients. It can be used for a selection of the next chemical compounds with the potential effect to repair damage in cells affected by the biallelic genetic defect. We hope that it will allow to obtain an innovative tool for further research on the pathogenesis and causal treatment of Wolfram syndrome.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Author Contributions

A.Z collected clinical and genetic data and wrote the draft of the manuscript. M.B. performed genetic analyses. E.P. performed in vitro studies. A.L. performed skin biopsies. W.M. performed statistical analysis and prepared the final version of the manuscript.

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