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The role of estrogen receptor status in proline dehydrogenase/proline oxidase-dependent apoptosis in breast cancer cells.

Doctoral dissertation in medical sciences

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ARTICLES INCLUDED IN THE DISSERTATION

The objective of Ph.D. dissertation is to evaluate the impact of estrogen receptor (ER) status on proline dehydrogenase/proline oxidase (PRODH/POX)-dependent apoptosis in breast cancer cells.

The research hypothesis was presented in a review paper:

P1. Lewoniewska, S., Oscilowska, I., Forlino, A., Palka, J. Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells. Biology, 2021, 10, 1314. MSWiA: 100 points, Impact Factor ISI: 5.079. DOI: 10.3390/biology10121314

The results of this study are presented in the research paper:

P2. Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone- Induced PRODH/POX- Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4.242. DOI: 10.3390/jcm10204641

Article type	**Number**	**Impact Factor**	**MNiSW points**
Articles included in the dissertation	2	9.321	240
Articles not included in the dissertation	14	9.320	460
Conference abstracts	13	-	-
Summary	29	18,641	700

ABBREVIATIONS

AMPK—	AMP-activated protein kinase
ATP—	Adenosine triphosphate
A-KG—	α- ketoglutaric acid
c-Myc—	Oncogene acting at the transcriptional and post-transcriptional level
DBD—	DNA-binding domain
DLD-1—	Colorectal adenocarcinoma cell line isolated from large intestine of a colon adenocarcinoma patient
DNA—	Deoxyribonucleic axid
E2—	Estradiol
ER—	Estrogen receptor
ERα—	Estrogen receptor α
ERβ—	Estrogen receptor β
ERE—	Estrogen response element
FoxA1—	Forkhead box protein A1
GAPDH—	Glyceraldehyde 3-phosphate dehydrogenase
GLU—	Glutamate
GLUT—	Glutamine
GLY—	Glycine
HIF-1α—	Hypoxia-inducible factor-1α

ICI-182-780—	Fulvestrant (anti-estrogen compound)
LBD—	Ligand binding domain
MCF-7—	Breast cancer cell line
MCF-7^{wt} cells—	Wild-type MCF-7 cells
MCF-$7^{shPRODh/POX}$ cells—	PRODH/POX-silenced MCF-7 cells
MDA-MB-231—	Breast cancer cell line
MDA-MB-231^{wt} cells—	Wild-type MDA-MB-231 cells
MDA-MB-$231^{shPRODH/POX}$ cells—	PRODH/POX-silenced MDA-MB-231 cells
Mir-23b—	Small non-coding RNA fragment
2-MOE—	2-methoksyestradiol
ORN—	Ornithine
oxLDLs—	Oxidized low density lipoproteins
P5C—	Δ1-pyrroline-5-carboxylic acid
P5CR—	Pyrroline-5-carboxylic acid reductase
P53—	Transcriptional factor
PARP—	Poly (ADP-ribose) polymerase
PIG-6 gene—	Gene encoding PRODH/POX
POX—	Proline oxidase
PPAR-γ—	Peroxisome proliferator-activated receptor gamma
PPP—	Pentose-phosphate pathway
PPRE—	Peroxisome proliferator response element

PRO—	Proline
PRODH—	Proline dehydrogenase
ROS—	Reactive oxygen species
RXR—	Retinoids X receptor
SERMs—	Selective ER modultors
TCA cycle—	Tricarboxylic acid cycle
TGZ—	Troglitazone
WT—	Wild type
X—	General symbol of amino-acid
X-PRO—	Amino acid-proline

INTRODUCTION

1. The mitochondrial function of proline dehydrogenase/proline oxidase (PRODH/POX)

Proline dehydrogenase (PRODH), also referred to as proline oxidase (POX), is a flavin nucleotide-dependent enzyme of mitochondrial origin [1, 2]. This enzyme catalyzes the conversion of proline to Δ1-pyrroline-5-carboxylic acid (P5C). During this process, electrons are transported to the respiratory chain contributing to the production of adenosine triphosphate (ATP) or reactive oxygen species (ROS). ATP production usually occurs under low glucose conditions. The generation of the ATP molecule replenishes energy deficits and promotes cell survival [3-5]. In the second case, ROS causes activation of caspase-9 and caspase-3, leading to apoptosis induction [3-7]. Likely, both processes (ATP production and ROS production) co-occur. However, the mechanism for directing the cell to the apoptosis or survival pathway is not fully understood. I have hypothesized that the high proline content and high PRODH/POX expression could accelerate ROS production, favoring induction of apoptosis.

2. The metabolic role of PRODH/POX

As mentioned in the previous paragraph, PRODH/POX catalyzes the conversion of proline to P5C. The reverse reaction, the conversion of P5C to proline, is catalyzed by pyrroline-5-carboxylic acid reductase (P5CR) [8,9]. PRODH/POX and P5CR co-form the so-called “proline cycle” (**Figure 1**). The “proline cycle” rate depends on the proline availability for PRODH/POX. The critical source providing substrate for PRODH/POX is prolidase. It is an enzyme that releases proline from imidodi- or imidotripeptides, the end product of collagen degradation [10,11]. The free proline formed in this reaction can be used as a substrate for PRODH/POX (the only proline degrading enzyme) or collagen biosynthesis (the primary process utilizing proline). However, a significant source of proline comes from other amino acids that are convertible into P5C as glutamine (GLUT), ornithine ((ORN) amino acid of urea cycle), and glutamate (GLU), which is the precursor of α-ketoglutaric acid ((α-KG) amino acid

of TCA cycle). The Pentose Phosphate Pathway (PPP) is also indirectly involved in proline metabolism [2, 12-16]. Since proline has a reducing potential, the proline pathway ensures a redox balance between the mitochondrion and the cytoplasm [6].

Proline also plays an essential role in regulating some transcription factors [17,18]. It has been shown that proline inhibits the degradation of hypoxia-inducible factor-1 (HIF-1α), facilitating its transcriptional activity. Increased PRODH/POX activity contributes to a decrease in proline availability for interaction with a specific domain of HIF-1α, leading to inhibition of the transcriptional activity of HIF-1α [19,20]. Therefore, factors inhibiting PRODH/POX expression increase proline concentration and HIF-1α transcriptional activity. Among such inhibitors of PRODH/POX are mir-23b and c-Myc (**Figure 1**). Mir-23b is a small non-coding RNA fragment [13, 21, 22]. It can bind directly to PRODH/POX mRNA. Overexpression of mir-23b leads to a decrease in PRODH/POX enzyme activity and blocks ROS production, thereby blocking apoptosis. The c-Myc is an oncogene acting at the transcriptional and post-transcriptional levels. It can potentiate the action of mir-23b [13, 21, 22]. However, the most potent factor regulating PRODH/POX activity is the p53 protein (transcriptional factor) **(Figure 1)** [23]. Among genes induced by p53 protein is the PIG-6 gene encoding PRODH/POX [24]. Therefore, P53 is involved in the transcriptional regulation of the enzyme expression. Another important PRODH/POX expression regulator is the peroxisome proliferator-activated receptor gamma (PPAR- γ). This issue is described in the next chapter.

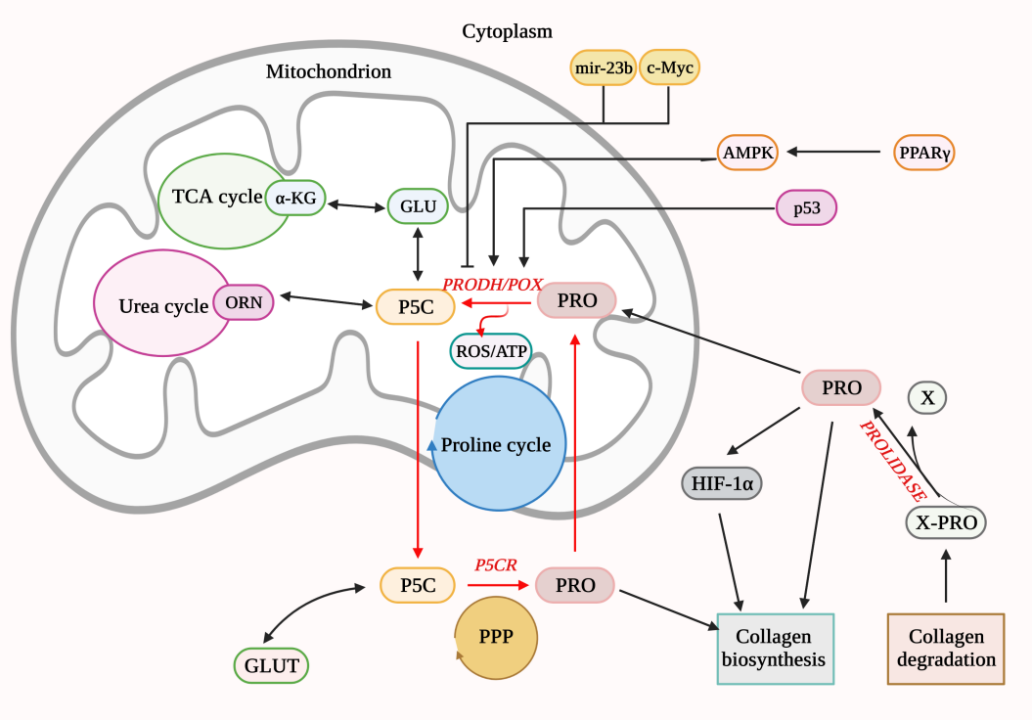

Figure 1. Complex regulatory mechanisms linking proline cycle, urea cycle, TCA cycle, pentose–phosphate pathway and collagen metabolism to PRODH/POX-dependent apoptosis/survival.

X-Pro—amino acid-proline; PRO—proline; X—amino acid; PRODH/POX—proline dehydrogenase/proline oxidase; P5C—Δ1-pyrroline-5-carboxylic acid; ROS—reactive oxygen species; ATP—adenosine triphosphate; GLU—glutamate; GLUT—glutamine; PPP—pentose-phosphate pathway; P5CR—pyrroline-5-carboxylic acid reductase; HIF-1α—hypoxia inducible factor 1α; TCA-cycle—tricarboxylic acid cycle; α-KG—α-ketoglutaric acid; ORN—ornithine; p53—transcriptional factor; AMPK—AMP-activated protein kinase; PPAR γ—peroxisome proliferator activated receptor gamma; Mir-23b—small non-coding RNA fragment; c-Myc—oncogene acting at the transcriptional and post-transcriptional level.

3. PPAR-γ inducing PRODH/POX expression and its role in apoptosis

The most potent factor stimulating PRODH/POX expression is the peroxisome proliferator-activated receptors (PPAR-γ), a nuclear hormone receptor and ligand-dependent transcription factor [25-27]. Activation of PPAR-γ results from ligand binding to the C-terminal region of the receptor and specifically to the ligand-binding domain (LBD), which alters the conformation of the receptor and enables its heterodimerization with retinoids X receptor (RXR) [28]. The complex translocates to the cell nucleus and fuses with the peroxisome proliferator response element (PPRE) [29]. The DNA-binding domain (DBD) is essential in this process. As a consequence, transcription of a specific gene is activated. PPRE is present in the PRODH/POX promoter sequence [29] and in the promoter sequence of the gene encoding cytochrome p450 and genes involved in lipid metabolism.

Endogenous and synthetic agonists represent PPAR-γ ligands. Their functions as PRODH/POX stimulators are different. Endogenous agonists of PPAR-γ are polyunsaturated fatty acids (arachidonic acid, linolenic acid) and their metabolites, prostaglandins, and oxidized low-density lipoproteins (oxLDLs) [29-31]. Activation of PRODH/POX with endogenous PPAR-γ activators has been shown to direct cells to the autophagy pathway, facilitating their survival [22]. Synthetic PPAR-γ agonists (e.g., thiazolidinediones) have activated ROS-dependent intrinsic apoptotic pathways through PRODH/POX activation [29,32]. In studies presented in the dissertation, the synthetic PPAR-γ agonist, troglitazone (TGZ), was used. It belongs to the group of antidiabetic and anti-inflammatory drugs [33-35]. Although TGZ is a discontinued drug, it was used as a model molecule in this study because it is a potent stimulator of PRODH/POX [36, 37]. The effect of TGZ on PRODH/POX expression and function is presented in **Figure 2.**

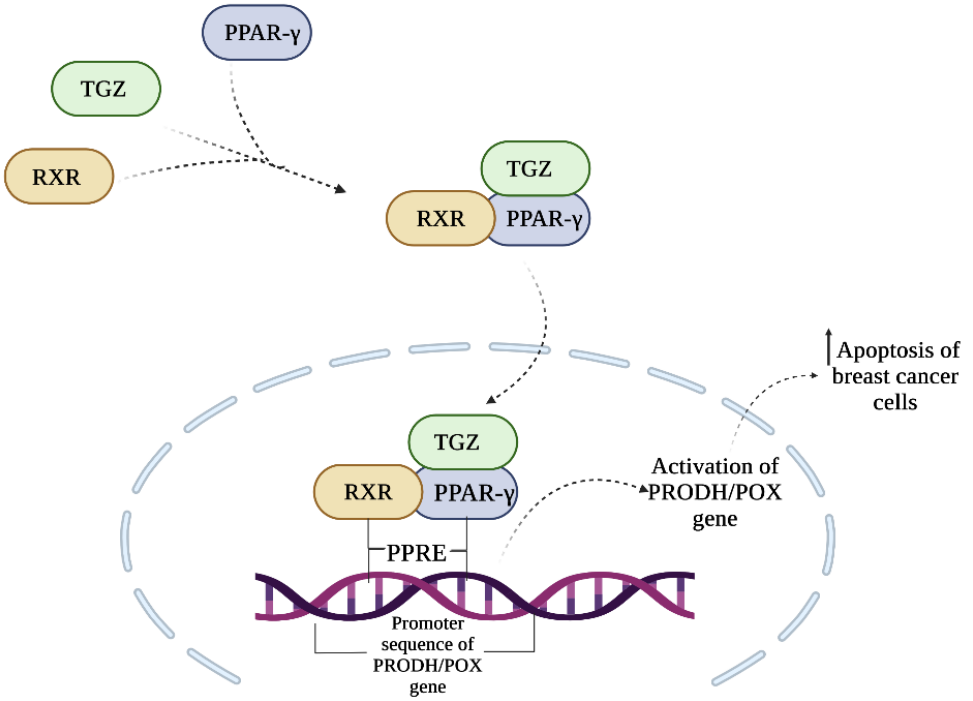

Figure 2. The effect of PPAR-γ agonist, TGZ on PRODH/POX expression and function.

TGZ—troglitazone; PPAR- y— peroxisome proliferator-activated receptor-gamma; RXR— retinoids X receptor; PPRE— peroxisome proliferator response element; PRODH/POX— proline dehydrogenase/proline oxidase.

4. The potential effect of estrogen receptor modulators on PRODH/POX-dependent functions.

Estrogens regulate cell proliferation and differentiation in various tissues [38-40]. The classical mechanism of a direct action of estrogens on gene expression involves the binding of estrogens to estrogen receptors (ERs), which then dimerizes with the estrogen-response element (ERE) via forkhead box protein A1 (FoxA1). This complex affects transcription and translation processes [41,42].

The functional activity of estrogens is dependent on the receptor status of a particular cell type. The estrogen receptors alpha (ERα) and beta (ERβ) differ in structure, distribution in tissues and organs, and biological activity [43-45]. It was suggested that ERα is involved in the regulation of cell proliferation, while ERβ is involved in anti-proliferative processes and creates the pro-apoptotic phenotype of cancer cells [46,47]. Therefore, ER status significantly impacts breast cancer cell growth and metastasis. For comparison, two breast cancer cell lines were studied ER-receptor positive cells (MCF-7- have both estrogen receptors ERα and ERβ) and ER-negative cells (MDA-MB-231-have only ERβ). It was found that in MCF-7 cells, estrogens enhance cancer cell proliferation while they respond well to anticancer treatment and are poorly metastatic [48]. Conversely, MDA-MB-231 cells show a solid metastatic potential [49].

Estrogens stimulate cell proliferation by upregulating AMP-activated protein kinase (AMPK) expression, stimulating PRODH/POX activity [50-51]. Estrogens regulate PRODH/POX activity at the level of ER, p53, HIF-1α, and collagen biosynthesis, which regulates the availability of the substrate (proline) for PRODH/POX [52, 53]. The link between PRODH/POX, ERα, and p53 was considered as a potential mechanism of apoptosis/survival regulation [54]. However, a significant role in PRODH/POX-dependent function is the availability of proline as a substrate for the enzyme. Estrogens were found to control the level of proline. They enhance collagen biosynthesis that utilizes proline, limiting its availability for PRODH/POX [55, 56]. An increase in collagen biosynthesis could facilitate therefore survival of breast cancer cells **(Figure 3)**.

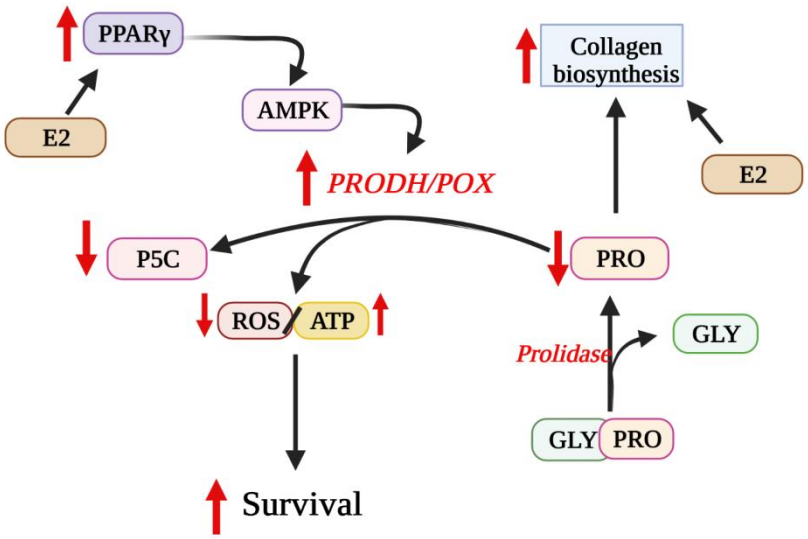

Figure 3. Graphical representation of the mechanism of estrogen-dependent regulation of PRODH/POX function.

P5C— Δ1-pyrroline-5-carboxylic acid; PRODH/POX—proline dehydrogenase/proline oxidase; ROS—reactive oxygen species; GLY—glycine; PRO—proline; PPAR γ—peroxisome proliferator activated receptor; ATP—adenosine triphosphate; PPARγ—peroxisome proliferator activated receptor gamma; AMPK—AMP-activated protein kinase; E2—estradiol.

Intriguing compounds of natural origin acting via ERs are phytoestrogens. More precisely, the group of isoflavones, e.g., biochanin A, genistein, and equol. It has been shown that isoflavones in low concentrations have estrogen-like effects as ER agonists [57]. In contrast, they act as ER antagonists at higher concentrations and show an anti-estrogenic effect [57]. Due to these characteristics, they are referred to as selective ER modulators (SERMs) [58]. Another compound showing anti-estrogenic activity is 2-methoxyestradiol (2-MOE). It is a metabolite of 17β-estradiol, and it has an opposite effect on this substrate [59, 60]. The strong effect of these compound as an inhibitor of collagen biosynthesis has been documented in the literature [59,60]. The graphical mechanism is shown in **Figure 4.** A more detailed description of all of the processes mentioned above can be found in the review paper included in this dissertation entitled:,, Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells".

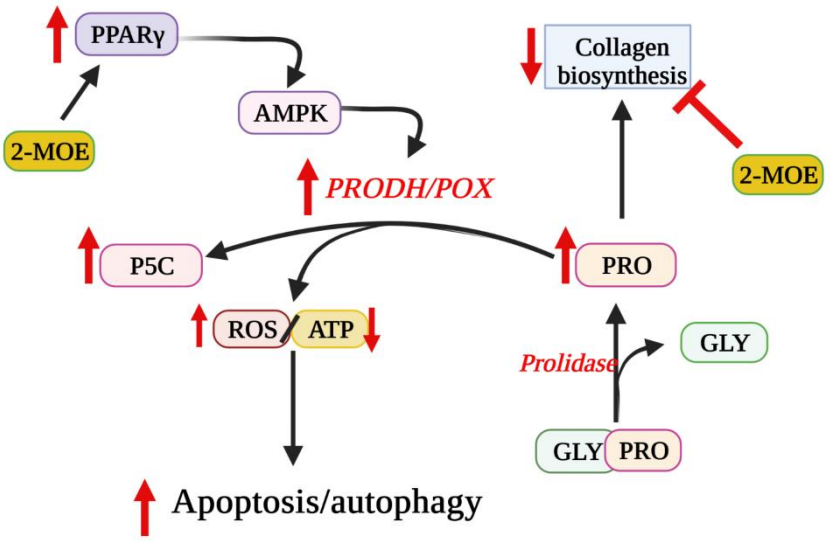

Figure 4. Graphical representation of the mechanism of action of antiestrogens using 2-MOE as an example.

2-MOE—2-methoxyestradiol; P5C— Δ1-pyrroline-5-carboxylic acid; PRODH/POX—proline dehydrogenase/proline oxidase; ROS—reactive oxygen species; GLY—glycine; PRO—proline; PPAR γ—peroxisome proliferator activated receptor gamma; ATP—adenosine triphosphate; AMPK—AMP-activated protein kinase.

OBJECTIVE OF THE STUDY

My scientific interest is focused on molecular targets of experimental breast cancer pharmacotherapy. Recent studies on mechanisms driving apoptosis in cancer cells highlighted the role of PRODH/POX in this process. PRODH/POX is a mitochondrial enzyme that catalyzes the conversion of proline to 1-pyrroline-5-carboxylic acid (P5C). During this conversion, electrons are transported to the respiratory chain, producing ATP, or they are directly accepted by oxygen, generating reactive oxygen species (ROS). In the first case, activation of PRODH/POX leads to the production of ATP for survival; in the second one, ROS induces apoptosis. Although the mechanism for the switch between apoptosis/survival is not well understood, it has been postulated that the PRODH/POX-induced apoptosis or survival is a metabolic context-dependent process, and proline availability for PRODH/POX-dependent functions may play a key role.

I have hypothesized that estrogens could play an essential role in the mechanism of PRODH/POX-dependent apoptosis/survival as stimulators of collagen biosynthesis that utilize a large amount of free proline, limiting substrate (proline) availability for PRODH/POX-dependent functions. Estrogens are implicated in collagen metabolism as stimulators of collagen biosynthesis. This process is accompanied by collagen degradation finalized by cytoplasmic imidodipeptidase, prolidase.

To explore the hypothesis, I have established two breast cancer cell models ER-positive breast cancer cell line (MCF-7 cells expressing ERα and ERβ) and ER-negative breast cancer cell line (MDA-MB-231 cells expressing only ERβ) with activated and shRNA silenced PRODH/POX. Since PPAR-γ is known to stimulate PRODH/POX, I used TGZ, PPAR-γ ligand, to up-regulate PRODH/POX as another cell line model.

The link between estrogens, collagen biosynthesis, PRODH/POX, proline, and apoptosis/survival in cancer cells allowed to present a hypothesis on the mechanism of estrogen-dependent regulation of PRODH/POX-dependent apoptosis. Whether ER status and ER ligands affect PRODH/POX-dependent apoptosis/survival is the aim of the study.

MATERIALS AND METHODS

The studies were performed on breast cancer cell lines: wild type MCF-7, wild type MDA-MB-231, PRODH/POX-silenced MCF-7, and PRODH/POX-silenced MDA-MB-231.

Wild type MCF-7 and wild type MDA-MB-231cells were obtained from ATCC (ATCC, Manassas, VA, USA). PRODH/POX-silenced MCF-7 and MDA-MB-231 cells were developed in the Department of Medicinal Chemistry, Medical University of Bialystok, as described previously [61]. Cells were incubated in the presence or absence of estradiol (2nmol/l), TGZ (10 or 20μmol/l), or both compounds in DMEM without phenol red.

DNA biosynthesis and collagen biosynthesis were evaluated by radiometric methods. Prolidase activity was measured by colorimetric assay. The expression of apoptotic markers was evaluated by Western-Immunoblot analysis. Proline concentration was measured by the LC-MS-Based Quantitative Analysis Method. Intracellular reactive oxygen species accumulation was measured using fluorescence assay.

All the methods, maintaining cell cultures, as well as the statistical analysis, are described in the research article (included in the dissertation) entitled ,,Troglitazone-Induced PRODH/POX- Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells".

RESULTS

This chapter presents the most important results published in the research paper (attached to the dissertation) entitled: „Troglitazone- Induced PRODH/POX- Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells".

1. Generation of PRODH/POX-silenced MCF-7 and PRODH/POX-silenced MDA-MB-231 breast cancer cells.

PRODH/POX expression was silenced by shRNA technology, as described previously [61]. In the research model, I created three silenced clones of MCF-7 and MDA-MB-231 cells, presented in **Figure 5.** Due to the best efficacy, I used clone 2 of both cell lines for further studies.

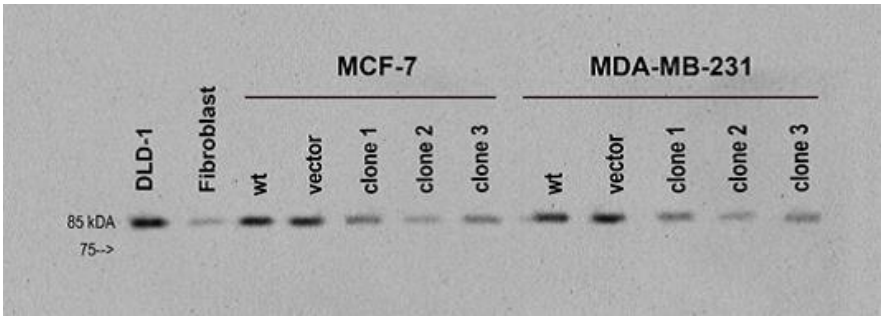

Figure 5. Efficacy of shRNA-based PRODH/POX knock-down in MCF-7 and MDA-MB-231 cells.
MCF-7— breast cancer cell line; MDA-MB-231—breast cancer cell line; wt—wild type; DLD-1—colorectal adenocarcinoma cell line.

2. TGZ-dependent inhibition of DNA-biosynthesis in wild type and PRODH/POX silenced MCF-7 and MDA-MB-231 cells.

In wild type MDA-MB-231 cells cultured in medium without estradiol, TGZ strongly inhibited deoxyribonucleic acid (DNA) biosynthesis (Figure 6D), while in the cells cultured in the presence of estradiol or in PRODH/POX silenced cells, the process was much less affected. Although the inhibition of DNA biosynthesis was also shown in

MCF-7 cells cultured with or without estradiol, the extent of inhibition was much lower than in MDA-MB-231 cells.

The data suggest that TGZ-induced inhibition of DNA biosynthesis is dependent on the presence of PRODH/POX and the absence of estradiol in MDA-MB-231 cells, expressing only ERβ.

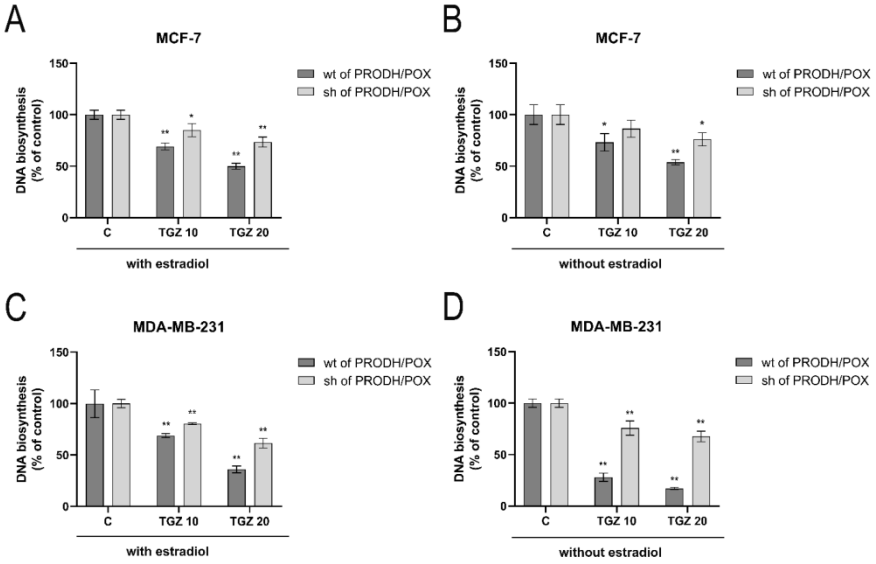

Figure 6. DNA biosynthesis in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 and MDA-MB-231 cells treated with troglitazone (TGZ) in the presence (A and C) and the absence (B and D) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks* indicate statistical differences between studied cells compared to controls at $P < 0.01$.

3. TGZ-dependent ROS production in wild type and PRODH/POX silenced MCF-7 and MDA-MB-231 cells.

In wild type MDA-MB-231 cells cultured in medium without estradiol ROS production was significantly increased (Figure 7H), while in the cells cultured in the presence of estradiol or in PRODH/POX silenced cells, the process was not affected. The effect was also not found in MCF-7 cells cultured either with or without estradiol. However, when ERβ was removed from MDA-MB-231 cells by ICI-182-780 (Fulvestrant)-dependent degradation, TGZ regardless of the absence or presence of estradiol in the medium also induced ROS production, suggesting that ERβ counteracts ROS production in MDA-MB-231 cells (Figure 7I, 7J). An increase in ROS production was accompanied by an increase in expressions of PPAR-γ and AMPK as demonstrated by Western blot (Figure 7E, 7F).

The data suggest that TGZ induces ROS production by PRODH/POX stimulated by PPAR-γ and AMPK.

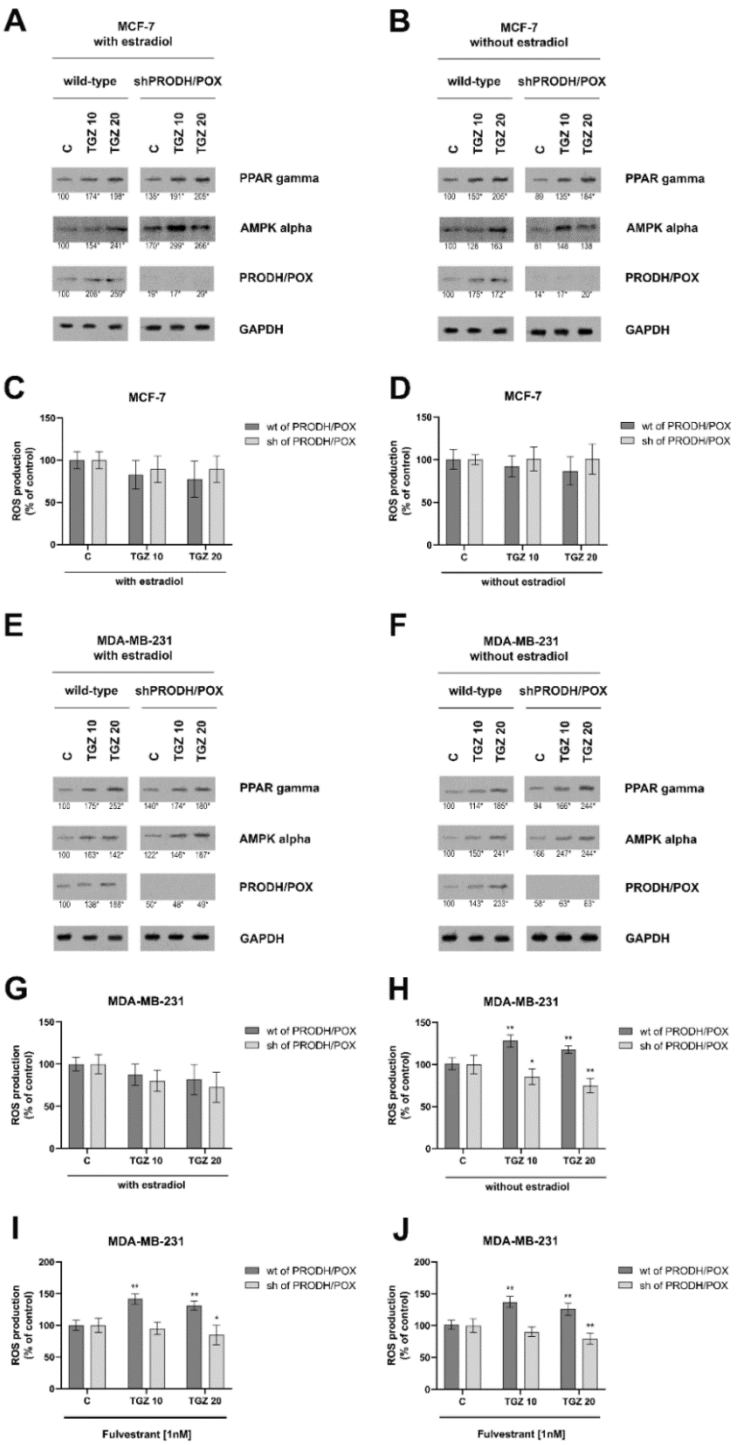

Figure 7. PRODH/POX, AMPK and PPAR-γ expressions in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 and MDA-MB-231 cells treated with troglitazone (TGZ) in the presence (7A and 7E) and absence (7B and 7F) of estradiol. The WB bands intensity of representative blots were quantified by densitometry and normalized to glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) (Supplementary Material, SFigure 2-SFigure 7). ROS production is presented in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 and MDA-MB-231 cells cultured with TGZ and/or fulvestrant (ICI 182 780) in the presence (7C, 7G and 7I) and the absence (7D, 7H and 7J) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks* indicate statistical differences between studied cells compared to controls at $P < 0.01$.

4. TGZ-dependent apoptosis in wild type and PRODH/POX silenced MCF-7 and MDA-MB-231 cells.

TGZ strongly induced expression of cleaved caspase-3, caspase-9, and poly (ADP-ribose) polymerase (PARP) in MDA-MB-231cells cultured in the absence of estradiol (Figure 8A, 8B). Although the effect was also shown in MCF-7 cells cultured with or without estradiol, the extent of inhibition was much lower than in MDA-MB-231 cells (Figure 9A, 9B).

An increase in the expression of studied caspases in TGZ-treated cells was accompanied by an increase in the expression of p53 particularly in MDA-MB-231 cells (Figure 8B) cultured in estradiol free medium.

It suggests that TGZ-dependent apoptosis in breast cancer cells is highly pronounced in ERβ expressing MDA-MB-231 cells cultured in estradiol-free medium.

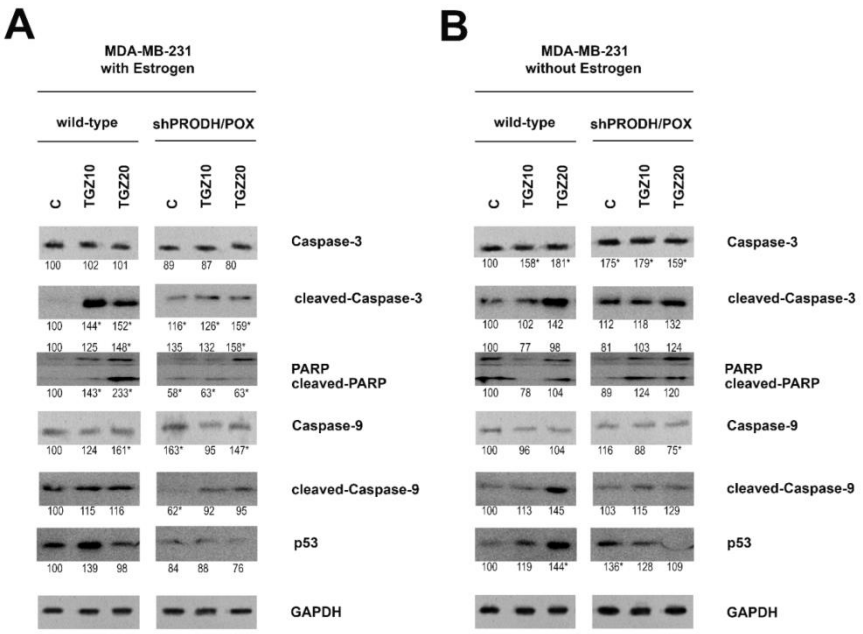

Figure 8. Western blot for cleaved caspase-3, PARP, caspase-9 and p53 in wild type and PRODH/POX-silenced (shPRODH/POX) MDA-MB-231 cells treated with troglitazone (TGZ) in the presence (A) and absence (B) of estradiol. GAPDH expression was used as a loading control. The Western blot bands intensity of representative blots were quantified by densitometry and normalized to GAPDH (Supplementary Material, SFigure 12-SFigure 15).

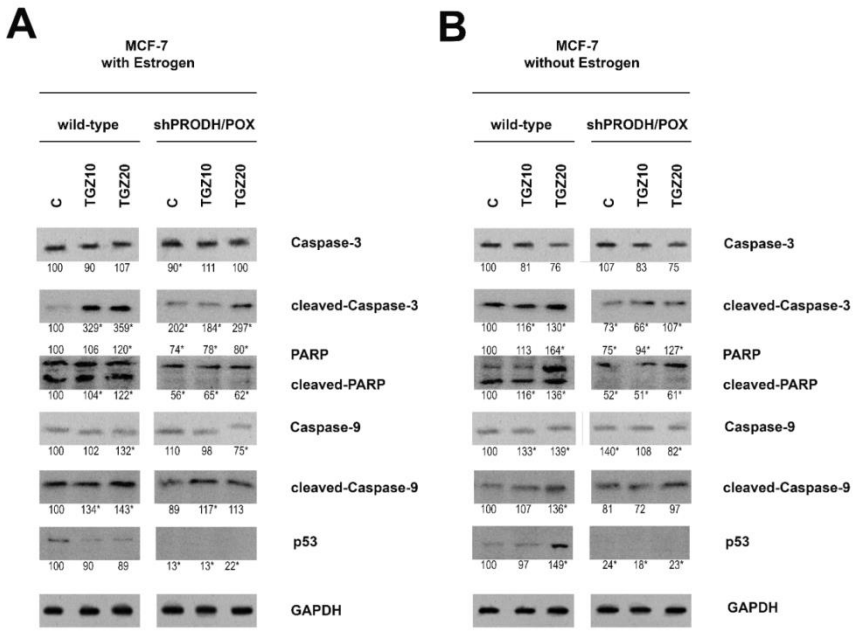

Figure 9. Western blot for cleaved caspase-3, PARP, caspase-9 and p53 in wild type and PRODH/POX-silenced (shPRODH/POX) MCF-7 cells treated with troglitazone (TGZ) in the presence (A) and absence (B) of estradiol. GAPDH expression was used as a loading control. The Western blot bands intensity of representative blots were quantified by densitometry and normalized to GAPDH (Supplementary Material, SFigure 8-SFigure 11).

5. TGZ increases proline availability for PRODH/POX via downregulation of collagen biosynthesis and up-regulation of prolidase activity in breast cancer cells.

PRODH/POX-induced apoptosis (through ROS generation) in breast cancer cells is dependent on proline availability. Intracellular free proline content is regulated mainly by collagen biosynthesis (proline utilizing process) and prolidase activity (proline releasing enzyme). In wild type and PRODH/POX-silenced MCF-7 cells cultured in the medium containing estradiol, TGZ induced a dose-dependent increase in proline concentration (Figure 10A) and inhibition of collagen biosynthesis (Figure 10C) and prolidase activity (Figure 10E). TGZ-dependent increase in proline concentration and

decrease in collagen biosynthesis and prolidase activity were more pronounced in PRODH/POX silenced cells. In MCF-7 cells cultured in the medium without estradiol TGZ contributed to an increase in proline concentration and a decrease in collagen biosynthesis in PRODH/POX-silenced cells while in wild type MCF-7 cells the processes were much less affected (Figure 10B, D). However, TGZ inhibited prolidase activity in wild type MCF-7 cells, while it had no significant effect on the enzyme activity in PRODH/POX silenced cells cultured in estradiol free medium (Figure 10F).

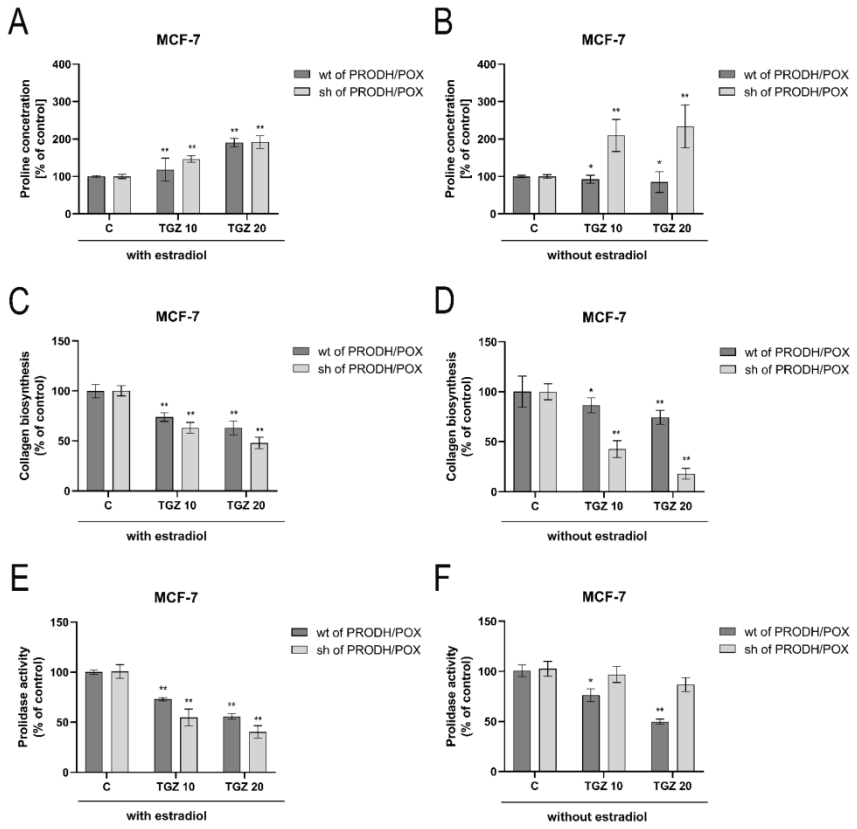

Figure 10. Proline concentration (A, B), collagen biosynthesis (C, D) and prolidase activity (E, F) in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 cells stimulated by troglitazone (TGZ) in the presence (A, C, E) and absence (B, D, F) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks* indicate statistical differences between studied cells compared to controls at $P < 0.01$.

In both wild type and PRODH/POX-silenced MDA-MB-231 cells cultured in medium containing estradiol, TGZ contributed to decrease in proline concentration (Figure 11A), collagen biosynthesis (Figure 11C) and prolidase activity (Figure 11E). The inhibition was less pronounced in PRODH/POX-silenced cells. In wild type MDA-MB-231 cells cultured without estradiol, TGZ contributed to dose-dependent decrease in proline concentration (Figure 11B), inhibition of collagen biosynthesis (Figure 11D) and prolidase activity (Figure 11F), while in PRODH/POX silenced cells, proline concentration and prolidase activity were not significantly affected and the inhibition of collagen biosynthesis was less pronounced than in MDA-MB-231 wild type cells (Figure 11B, 11D, 11F). It suggests that TGZ-induced inhibition of collagen biosynthesis could facilitate proline availability for PRODH/POX-dependent functions.

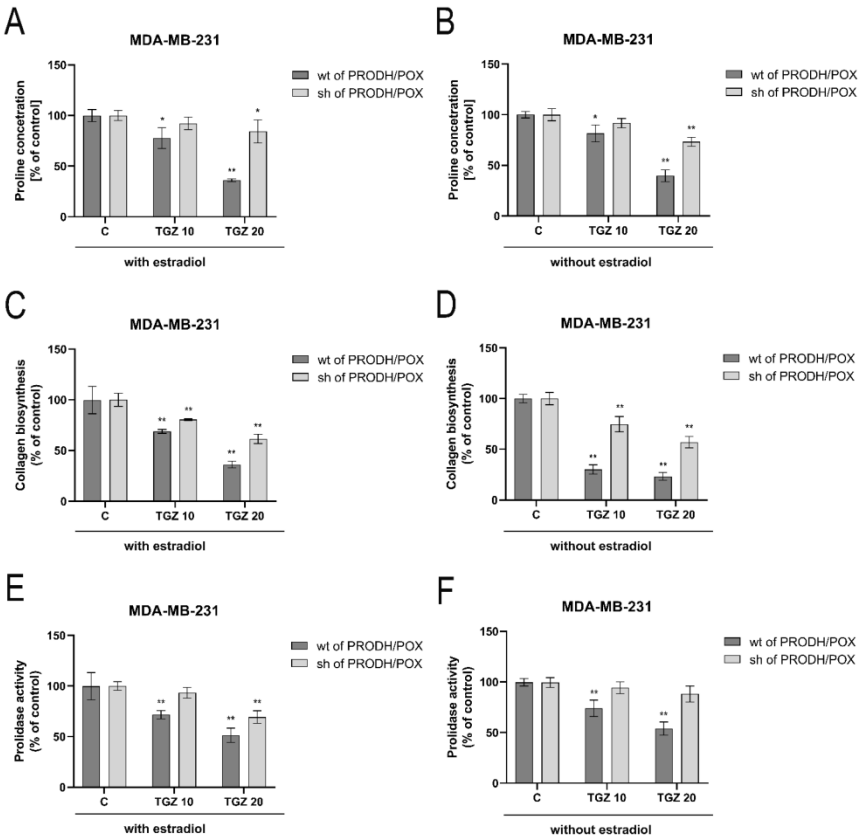

Figure 11. Proline concentration (A, B), collagen biosynthesis (C, D) and prolidase activity (E, F) in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MDA-MB-231 cells stimulated by troglitazone (TGZ) in the presence (A, C, E) and absence (B, D, F) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks* indicate statistical differences between studied cells compared to controls at $P < 0.01$.

DISCUSSION

This chapter is discussed in details in the research paper:

P2. Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone-Induced PRODH/POX-Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4,242. DOI: 10.3390/jcm10204641.

CONCLUSIONS

1. The PRODH/POX silenced MCF-7 and MDA-MB-231 breast cancer cells generated by shRNA technology enable an analysis of the functional significance of PRODH/POX in apoptosis/survival in breast cancer cells.
2. Troglitazone (TGZ), the ligand of PPAR-γ, induces PRODH/POX expression and inhibits collagen biosynthesis in breast cancer cells.
3. TGZ strongly induces PRODH/POX-dependent apoptosis in MDA-MB-231 cells cultured in the medium without estradiol or deprived of ERβ. The apoptosis is mediated by PRODH/POX-dependent ROS generation and the process is facilitated by proline availability for PRODH/POX by TGZ-dependent inhibition of collagen biosynthesis.
4. The results suggest that combined PPAR-γ agonist and anti-estrogen treatment could be considered in experimental therapy of ER-negative breast cancers.

PUBLICATION 1

P1. Lewoniewska, S., Oscilowska, I., Forlino, A., Palka, J. Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells. Biology, 2021, 10, 1314. MSWiA: 100 points, Impact Factor ISI: 5.079. DOI: 10.3390/biology10121314.

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Review

Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells

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Simple Summary: The estrogen receptor (ER) status and the availability of agonists or antagonists of these receptors determine the processes of growth, differentiation, and proliferation of breast cancer cells. Estrogens and anti-estrogenic compounds have been shown to influence breast cancer cell survival/apoptosis via action through the mitochondrial enzyme proline dehydrogenase/proline oxidase (PRODH/POX). In this review, we highlight the molecular effects of ER stimulation/inhibition in signaling pathways.

Abstract: It has been suggested that activation of estrogen receptor α (ER α) stimulates cell proliferation. In contrast, estrogen receptor β (ER β) has anti-proliferative and pro-apoptotic activity. Although the role of estrogens in estrogen receptor-positive breast cancer progression has been well established, the mechanism of their effect on apoptosis is not fully understood. It has been considered that ER status of breast cancer cells and estrogen availability might determine proline dehydrogenase/proline oxidase (PRODH/POX)-dependent apoptosis. PRODH/POX is a mitochondrial enzyme that converts proline into pyrroline-5-carboxylate (P5C). During this process, ATP (adenosine triphosphate) or ROS (reactive oxygen species) are produced, facilitating cell survival or death, respectively. However, the critical factor in driving PRODH/POX-dependent functions is proline availability. The amount of this amino acid is regulated at the level of prolidase (proline releasing enzyme), collagen biosynthesis (proline utilizing process), and glutamine, glutamate, α-ketoglutarate, and ornithine metabolism. Estrogens were found to upregulate prolidase activity and collagen biosynthesis. It seems that in estrogen receptor-positive breast cancer cells, prolidase supports proline for collagen biosynthesis, limiting its availability for PRODH/POX-dependent apoptosis. Moreover, lack of free proline (known to upregulate the transcriptional activity of hypoxia-inducible factor 1, HIF-1) contributes to downregulation of HIF-1-dependent pro-survival activity. The complex regulatory mechanism also involves PRODH/POX expression and activity. It is induced transcriptionally by p53 and post-transcriptionally by AMPK (AMP-activated protein kinase), which is regulated by ERs. The review also discusses the role of interconversion of proline/glutamate/ornithine in supporting proline to PRODH/POX-dependent functions. The data suggest that PRODH/POX-induced apoptosis is dependent on ER status in breast cancer cells.

Keywords: estrogens; advanced cancer; estrogen receptor; breast cancer

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1. Introduction

According to the WHO (World Health Organization) epidemiological data, for 2020, 19.3 million people developed cancer, and about 10 million people died. Breast cancer was

the most common malignant neoplasm in women and accounted for 11.7% of all cancers globally. WHO cites obesity as one of the main reasons for the high incidence of the disease. The recent increase in the mortality of breast cancers was due to the COVID-19 pandemic that affected both therapy and prevention of the disease [1,2]. Although several therapeutic approaches for breast cancer treatment have been established, the role of estrogen receptor (ER) status in the complex regulatory mechanisms driving apoptosis/survival of cancer cells is not fully understood. Most of the studies presented in this review were done on breast cancer cell models. Although cell line models have some limitations (e.g., inability to observe systemic phenomena), they are a powerful tool which offers several advantages. Certainly, the cell models allow to strictly control conditions of the experiment in order to establish the critical factor affecting the studied processes. They are especially helpful in case of limited availability of clinical samples or in vivo models (e.g., estradiol deficiency or estrogen receptor status). Therefore, results on cell models allow to predict the consequences of pharmacotherapeutic manipulation in human. Different treatment regimens and combinations of therapies have been tested using cell lines which have yielded interesting and potentially promising results that currently have an application value [3,4].

The presence of the ER (ER+) in breast cancers increases positive response to anticancer treatment. Moreover, a better prognosis concerns progesterone receptors (PR+) and human epidermal growth factor (HER2+) positive cancers. The absence of ER is a significant risk factor for relapse and shorter life expectancy. Some authors emphasize that at least a two-receptor ER+PR+HER- expansion profile has a better prognosis than a single-receptor profile such as ER+PR-HER- or ER-PR+HER- [3]. This is probably due to the hormonal reorganization of cellular metabolism driving pro-survival or pro-apoptotic pathways. However, the mechanisms driving apoptosis/survival are not fully understood. In this report, we provide evidence that some of the ER functions could be attributed to proline dehydrogenase/proline oxidase (PRODH/POX).

2. Estrogen Receptors Structure, Location and Function

Two distinct estrogen receptor (ER) types, ERα and ERβ, are known to be encoded by two different genes located on two different chromosomes. ERα and ERβ are encoded by ESR1 (chromosome 6, region q24-q27) and ESR2 gene (chromosome 14, region q23.2). The molecular weight of ERα is 67 kDa, the ERβ isoform has 57 kDa [4]. Both types are composed of 6 functional domains named A–F [5]. Domains A and B are located at the amino terminal of the protein. The domain AF1 is able to activate gene transcription in the absence of bound ligand (e.g., the estrogen); however, the activation is weak. Domain C is responsible for receptor dimerization and binding of the ligand-receptor complex to a specific sequence on DNA. The D domain is also called the hinge. It has DNA-binding properties, and its sequence is more variable than that of the C domain. Next is the E domain, which contains a hydrophobic pocket structure called the ligand-binding domain (LBD). The E domain also enables dimerization of nuclear receptors. Some receptors also have an F domain, whose role is not fully elucidated (Figure 1) [5].

Non-active ERs occur in the cell cytosol, where they form large complexes with chaperone proteins of the HSP (Heat Shock Proteins) family. In this form, they are still inactive but capable of ligand attachment [5]. Ligand binding causes dimerization of the receptor. This process is crucial for the formation of a functional transcription factor and the regulation of gene transcription interacting with the Estrogen Response Element (ERE) (Figure 2). The molecule required for the binding of ER to DNA is FoxA1. It is a critical factor that promotes binding to chromatin [6].

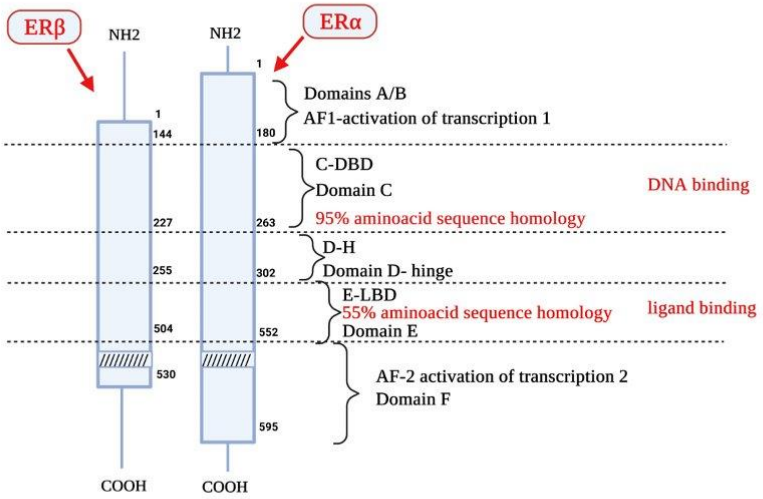

Figure 1. The structure of the estrogen receptor. ERα—Estrogen Receptor α; ERβ—Estrogen Receptor β; AF1—activator of transcription 1; C-DBD—DNA Binding Domain, domain C; D-H—Domain D-hinge; E-LBD—Ligand Binding Domain, domain E; AF-2—activator of transcription 2; NH2—amino-terminus, NH2—terminus, N—terminal end or amine-terminus; COOH—carboxylic terminus.

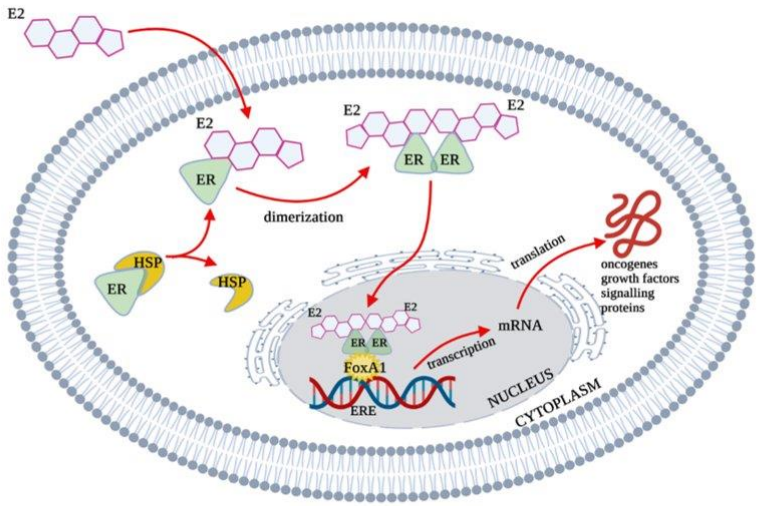

Figure 2. ER-dependent gene transcription. E2—estradiol; ER—Estrogen Receptor, HSP—Heat Shock Proteins; FoxA1—Forkhead box protein A1; ERE—Estrogen Response Element; mRNA—messenger RNA.

The distribution of ERα and ERβ receptors in tissues and organs varies. In most tissues and organs, both types of estrogen receptors are present, while in some, only one type predominates. In the ovaries, uterus, mammary gland, kidney, adrenal gland, testes, epididymis, pituitary gland, and hypothalamus, ERα expression is higher [7–9] than in the urinary bladder, prostate gland, heart, and liver [10]. The highest level of ERβ expression

was found in the ovary and prostate gland [11]. An important function of estrogen receptors is transcriptional and post-transcriptional regulation of cellular metabolism [12]. It has been suggested that ERα is involved in the regulation of cell proliferation, while ERβ evokes anti-proliferative and pro-apoptotic activity [13,14]. However, ERs comprise also several membranes bound receptors as G protein-coupled estrogen receptor (GPER) and Gq-coupled membrane estrogen receptor (GqmER). Recent studies revealed a functional link between all types of ERs. Interestingly, several oncogenic miRNAs have been shown to modulate the expression of ERs affecting malignant behaviour of cancer cells [15]. Moreover, a ligand-independent signaling has been reported for ERα through kind of cross-talk with epidermal growth factor or insulin-like growth factor-I [16,17]. Whether they are involved in PRODH/POX-dependent regulation of apoptosis/survival requires to be explored.

3. Apoptosis

Apoptosis is the process of programmed cell death, important in the development and homeostasis of multicellular organisms [18]. This process enables the elimination of damaged, old or unnecessary cells. Initiation of the apoptosis pathway is one of the possible cell responses to intracellular or extracellular action of the chemical, physical or biological factors. The external factors that cause cell damage include UV radiation, ionizing radiation, thermal shock, low availability of oxygen and nutrients, drugs, or viral and bacterial infections [19]. The internal factors are activated by oncogenes, cell cycle defects, deficiency of growth factors, energy, hormonal deregulation, etc. [20,21]. Factors inducing apoptosis contribute to the development of neurodegenerative and autoimmune diseases, growth defects, and cancer. The disturbed balance between survival and apoptosis is a common feature of cancer cells [22]. It is also the cause of resistance to chemotherapy, radiotherapy, hormonal and immune therapy [23].

Apoptosis is a precisely regulated process by several classes of proteins. The most important are caspases (a family of intracellular cysteine proteases). They are divided into initiator, implementing, and inflammation caspases. Another important protein in the apoptosis process is the family of BCL-2 proteins (Bax; Bak, Bid, Bim), which have proapoptotic, antiapoptotic, and regulatory activities [24].

Several pathways lead to the induction of apoptosis. The extrinsic pathway is initiated by binding a ligand to the death surface receptors [25]. The intrinsic pathway of apoptosis can be activated by proapoptotic factors released from mitochondria. Apoptogenic molecules that are produced during intracellular stress leads to the increase in permeation of mitochondria. Both pathways stimulate apoptosis through proteolytic cleavage of procaspases into active enzymes [26]. The initiator caspases include caspase-8, -9, -10, whereas caspases-3, -6, and -7 are called effector caspases [27]. They can disrupt entire cells within a few minutes.

3.1. The Extrinsic Apoptosis Pathway

The extrinsic process of apoptosis is induced in the cell through the signals from other cells activating the death receptor, which initiates a cascade of intracellular effector proteins [28,29]. Tumor necrosis factor (TNF) is the best-characterized protein that initiates programmed cell death [30]. The same superfamily includes ligand of TNF family receptors (THANK), lymphotoxin (LT), Fas Ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), or the Vascular Endothelial Growth Inhibitor (VEGI) [31]. Some of them contain an intracellular death domain (DD). During protein binding to the receptors of the TNF family, the TRADD (Tumor necrosis factor receptor type 1-associated DEATH domain protein) or FADD (Fas-associated protein with death domain) adapter proteins interact with the DD region. Subsequently, the DISC complex (Death-inducing signaling complex) is formed [32–34]. This complex combines procaspases -8 and -10 and has autoproteolytic activation properties [35]. Cleaved caspases -8 and -10 activate the implementing caspases and initiate changes in the cell structure leading to cell death [32]. In addition, active

caspases -8 and -10 activate BID (a pro-apoptotic BCL family protein), which leads to increased release of cytochrome C from mitochondria by its truncated form tBID (Figure 3).

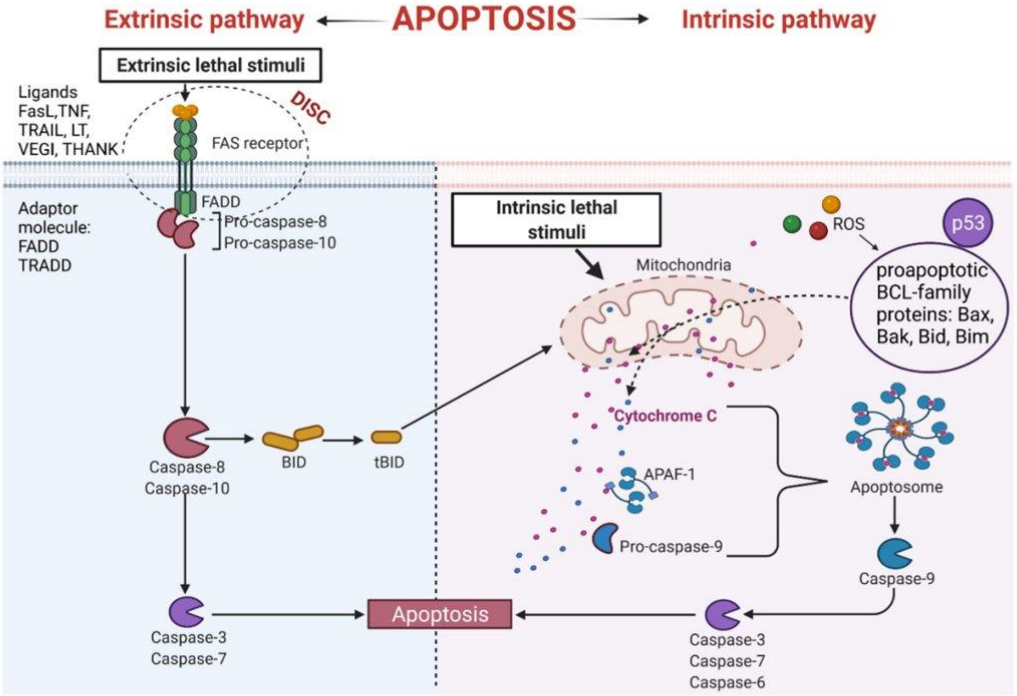

Figure 3. Intrinsic and extrinsic apoptotic pathway. THANK—TNF family receptor; LT—lymphotoxin; FasL—Fas Ligand; TRAIL—TNF-related apoptosis-inducing ligand; VEGI—Vascular Endothelial Growth Inhibitor; TNF—tumor necrosis factor; DISC—Death-inducing signaling complex; FADD—Fas-associated protein with death domain); TRADD—tumor necrosis factor receptor type 1-associated DEATH domain protein; p53—tumor protein p53; APAF-1—apoptotic protease activating factor-1; ROS—reactive oxygen species; Bax, Bak, Bid, Bim—proapoptotic BCL-family proteins; tBID—truncated BID.

An important apoptosis inducer is a p53 protein. This protein participates in the external and internal pathways of apoptosis. p53 interacts with BCL (B-cell lymphoma) proteins family contributing to the upregulation of mitochondrial channels and the cytochrome C efflux into the cytoplasm, activating the internal pathway of programmed cell death [36,37]. It has been established that p53 also induces genes coding for death receptors and death ligands [37].

3.2. The Intrinsic Apoptosis Pathway

This pathway is also called a mitochondrial pathway. It depends on energetic and metabolic processes in the cells and is induced by stress factors. These factors are oxidative stress, DNA damage, changes in cytoplasmic calcium ions concentration, and others. Furthermore, the production of reactive oxygen species (ROS) activates pro-apoptotic BCL- family proteins [38]. As a result of these reactions, the mitochondrial membrane is leaking [39], leading to the release of cytochrome C from mitochondria [38]. Released cytochrome C binds with procaspase9 and apoptotic protease activating factor-1 (APAF-1), forming apoptosome complex. The complex activates the cascade of structural changes

in the cell that contribute to cell death through active forms of executive caspases such as caspase-3, caspase-6, and caspase-7 (Figure 3) [40,41].

4. Functional Significance of PRODH/POX in Cell Metabolism

Proline oxidase (POX), also known as proline dehydrogenase (PRODH), is a mitochondrial flavin enzyme associated with the inner mitochondrial membrane. The enzyme catalyzes proline degradation by converting this amino acid to Δ1-pyrroline-5-carboxylic acid (P5C). During this reaction, electrons are transferred via flavin adenine dinucleotide (FAD) to cytochrome C in the respiratory chain, producing ATP molecules, facilitating survival. However, when electrons are transferred directly to oxygen, that happens in specific metabolic conditions, ROS are formed, inducing apoptosis or autophagy [42–45].

Although the mechanism for switching from ATP to ROS production is not fully understood, it has been suggested that excessive rates of electron transport may contribute to ROS generation [46]. The mechanism of this process is based on mitochondrial membrane potential driving ATP synthase and ATP production and the Kadenbach mechanism (occurring at high ATP/ADP radio) that involves binding of ATP to cytochrome c oxidase (CytOx) and inhibition of the enzyme. In stress situation, ATP-dependent inhibition is switched off and CytOx activity is determined by membrane potential leading to an increase in ROS production. Another mechanism depends on the quantity of electron transfer to the Heme aa3 of CytOx and, in case CytOx is inhibited by ATP, ROS production is decreased. Whether PRODH/POX-dependent ATP/ROS generation involves the same mechanism requires to be explored. However, it has been found that PRODH/POX binds to Coenzyme Q1 (coQ1) decreasing respiratory fitness that was counteracted by N-acetyl-cysteine, suggesting that the effect was mediated by PRODH/POX-dependent ROS formation [47]. Of interest is also finding that PRODH/POX is inhibited by succinate alleviating PRODH/POX effects on respiratory fitness. It suggests that PRODH/POX-induced ATP or ROS formation is metabolic contextdependent.

Conversion of mitochondrial proline into P5C by PRODH/POX may contribute to ROS-dependent intrinsic and extrinsic apoptosis [45,48–52]. It has been well established that overexpression of PRODH/POX causes cytochrome C release from mitochondria to cytosol and activation intrinsic apoptotic pathway by caspases-3 and -9 [53]. However, it has been also shown that upregulation of PRODH/POX induces caspase-8 activation in the extrinsic apoptotic pathway through stimulation of TNF-related apoptosis inducing ligand (TRAIL) and death receptor 5 (DR5) [53]. Moreover, the mechanism of PRODH/POX-dependent apoptosis may involve modulation of cell signaling pathways and cell cycle regulatory processes that could induce extrinsic apoptosis. The most potent inducer of PRODH/POX activity is tumor suppressor p53. Transcriptional regulation of PRODH/POX by p53 was found in the PRODH/POX promoter, containing a p53-response element [54–56].

It seems that ATP or ROS generation depends on the metabolic context in which proline availability for PRODH/POX and proline utilization processes play a critical role. Prolidase is an important factor in providing substrate for PRODH/POX. This enzyme catalyzes the last stage of collagen degradation by releasing proline or hydroxyproline from the C-terminus of imidodi- or imidotripeptides [57,58]. Free proline could be degraded by PRODH/POX or reused for collagen biosynthesis [59]. Proline for PRODH/POX could be also derived from amino acid metabolism. The most important are glutamate and ornithine yielding P5C in reactions catalyzed by P5C synthase and ornithine aminotransferase, respectively. The generated P5C is converted into proline in reaction catalyzed by isoforms of P5CR (P5C reductases). The conversion of glutamate to the proline is catalyzed by mitochondrial PYCR $\frac{1}{2}$, while the conversion of ornithine to proline is catalyzed by cytosolic PYCRL that is coupled to the Pentose Phosphate Pathway (PPP). PPP maintains a redox balance between cytosol and mitochondrion and participates in the synthesis of nucleotides [45,48]. However, the proline conversion product, P5C, can be rapidly used to synthesize glutamate by P5CDH (P5C dehydrogenase). Glutamate is, in turn, a precursor

for the synthesis of α-ketoglutaric acid, which is a component of the tricarboxylic acid cycle (TCA) [48]. Proline can also be converted to ornithine, which in turn is a component of the urea cycle. These reactions link TCA and urea cycles with amino acids metabolism determining proline availability for PRODH/POX-dependent functions (Figure 4). However, the enzyme could be regulated by other factors. An important transcriptional regulator of PRODH/POX is the p53 protein. The presence of a response element for p53 protein in the promoter sequence of the gene coding PRODH/POX has been demonstrated. It indicates the direct participation of p53 in the transcription of PRODH/POX [49]. Among factors that inhibit the expression of PRODH/POX is the oncogenic c-MYC transcription factor that may indirectly affect PRODH/POX by stimulating expression of PRODH/POX-inhibiting factor–miR-23b [50]. This is an endogenous, non-coding small RNA fragment that has the ability to bind to the PRODH/POX 3′UTR mRNA. It has been shown that overexpression of miR-23b resulted in downregulation of PRODH/POX expression [52].

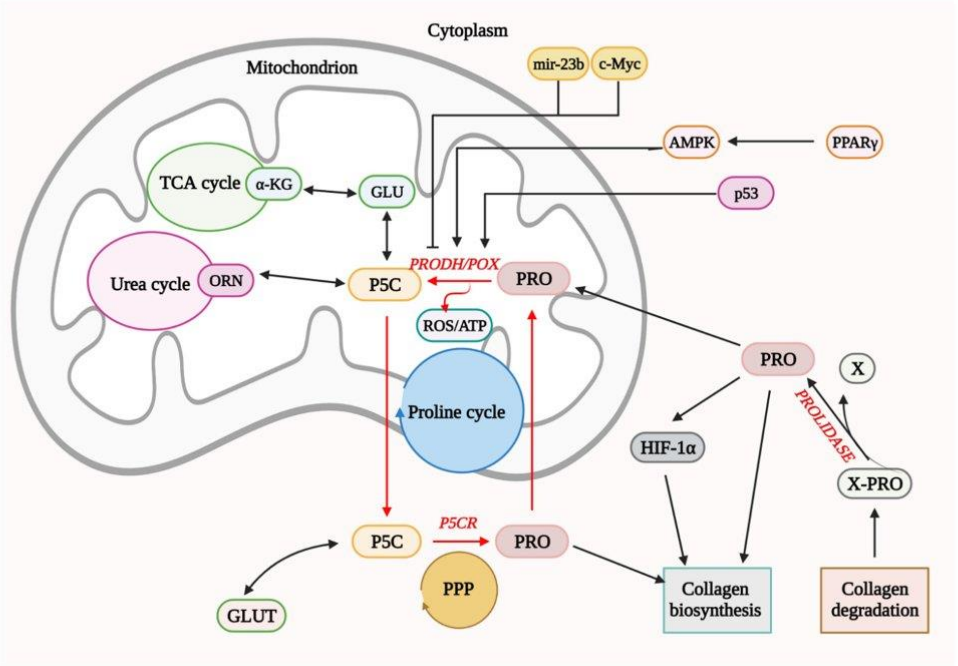

Figure 4. Complex regulatory mechanisms linking proline cycle, urea cycle, TCA cycle, pentose–phosphate pathway and collagen metabolism to PRODH/POX-dependent apoptosis/survival. X-Pro—amino acid-proline; PRO—proline; X—amino acid; PRODH/POX—proline dehydrogenase/proline oxidase; P5C—pyrroline-5-carboxylate; ROS—reactive oxygen species; ATP—adenosine triphosphate; GLU—glutamate; GLUT—glutamine; PPP—Pentose-Phosphate Pathway; P5CR—P5C reductase; HIF-1α—Hypoxia inducible factor 1α; TCA-cycle—The tricarboxylic acid cycle, also known as the Krebs or citric acid cycle; α-KG—α-ketoglutaric acid; ORN—ornithine; p53—TP53 or tumor protein; AMPK—AMP-activated protein kinase; PPAR γ—peroxisome proliferator activated receptor.

The best characterized PRODH/POX expression inducer is the peroxisome proliferator-activated receptor γ (PPAR-γ). It was shown that in the promoter sequence of the gene-encoding PRODH/POX, there are regions binding ligand-activated receptors, the so-called PPRE or PPAR-γ response element [51].

PRODH/POX participates in the induction of apoptosis by activating both extrinsic and intrinsic pathways. Activation of the extrinsic pathway requires stimulation of the transmembrane receptors containing the death domain through specific ligands [53]. PRODH/POX activates the extrinsic pathway via stimulation of geminin production. This action leads to cell cycle arrest in the G2 phase and cell apoptosis [55]. Moreover, PRODH/POX stimulates DNA damage inducible genes (GADDs).

PRODH/POX also participates in apoptosis by activating the intrinsic pathway (Figure 4). ROS formed during PRODH/POX-induced proline degradation disrupt transport in the mitochondrial membrane and affect membrane potential. In consequence, cytochrome C is released from the intermembrane space and initiates the intrinsic apoptosis pathway [54].

PRODH/POX indirectly inhibits the process of angiogenesis in tumor tissues and thus tumor growth. It degrades proline that was shown to upregulate the transcriptional activity of HIF-1α (Hypoxia-inducible factor-1) and HIF-1-dependent proteins such as vascular endothelial growth factor (VEGF) [56,60,61]. It has been documented that, in standard conditions, HIF-1α is continuously degraded by pVHL (Hippel–Lindau tumor suppressor gene product), which is a mediator in the ubiquitin pathway [56]. Proline has been shown to inhibit the degradation of HIF-1α, increasing its transcriptional activity [60]. Therefore, degradation of proline by PRODH/POX contributes to a decrease in proline level, expression of HIF-1, and angiogenesis [61].

PRODH/POX also participates in the regulation of cell proliferation. This enzyme, together with P5C reductase, forms proline cycle coupled to PPP-producing nucleotides for DNA biosynthesis (Figure 4) [62,63].

5. Involvement of ER Agonists in PRODH/POX-Dependent Apoptosis

ERs regulate the expression of AMP kinase (AMPK), which stimulates the activity of PRODH/POX [64,65].

The primary ligands for ER are estrogens, which represent a group of pleiotropic hormones. There are two dominant sources of estrogens in female physiology. In the pre-menopausal age, the ovaries are the principal producer of estrogens. In the post-menopausal age, when ovarian estrogen production declines, fat tissue becomes the main source. Adipocytes have a specific enzyme called aromatase, which converts testosterone to estrogen [66]. ER ligands—estrone, estriol, estradiol, and 2-hydroxy estrone—play functional roles in the physiology of the central nervous system, bones, reproductive and cardiovascular system. However, they also play an important role in carcinogenesis, stimulating cancer cell growth. These hormones act on the cancer cells by targeting the steroid receptor complex to specific DNA sequences, activating specific gene transcription. Several studies have demonstrated this mechanism using tamoxifen, a selective estrogen receptor modulator that inhibits estrogen-dependent tumor growth [67].

Estrogens regulate PRODH/POX-dependent functions at the level of ER, p53, substrate availability for PRODH/POX that is dependent on prolidase activity (proline supporting enzyme) and collagen biosynthesis (proline utilizing process), as well as HIF-1α. It seems that the most important player in determining pro-apoptotic/anti-apoptotic phenotype of cancer cells is the correlation between ERα, P53, and PRODH/POX. As pointed out in the above section, PRODH/POX is a P53-induced gene promoting apoptosis. However, ERα antagonizes P53-dependent apoptosis, promoting cell survival [68–70]. Based on these data, it has been established the mechanism for ERα anti-apoptotic potential, suggesting the formation of ERα-P53 complex [71]. Since ERβ was found to attenuate the complex formation, it was concluded that ERβ has pro-apoptotic activity [71]. Whether pro-apoptotic activity of ERβ undergoes through PRODH/POX that has either pro-apoptotic or pro-survival potential requires further study.

Another potential link between estrogens and PRODH/POX-dependent apoptosis is at the level of substrate availability for the enzyme. PRODH/POX is the only enzyme that degrades proline. During this process, ATP or ROS are produced (Figure 4). This

amino acid could be synthesized from glutamine or ornithine. However, it is energetically unfavorable, particularly in cancer cells. Instead, proline is derived from collagen degradation products. The last step of collagen degradation is catalyzed by prolidase, releasing proline from imododipeptides [63]. The activity of prolidase may regulate proline availability for PRODH/POX-dependent functions. However, the free proline could be rapidly used for collagen resynthesis, limiting its degradation by PRODH/POX in mitochondria. Such a case may take part in MCF-7 breast cancer cells, where estradiol (independently on the ERβ/ERα status) was found to stimulate collagen biosynthesis (Figure 5a) [72–74]. This process limits proline availability to the proline cycle (Figure 4) and PRODH/POX-dependent functions. It has been found that ERα is involved in the upregulation of prolidase activity, suggesting that it supports proline for collagen biosynthesis [75]. Interestingly, it also induces HIF-1α transcriptional activity, contributing to the pro-survival phenotype of breast cancer cells [75].

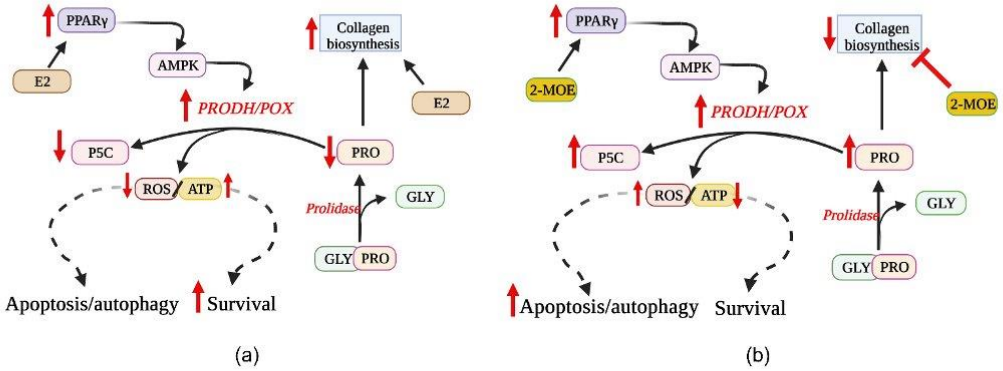

Figure 5. The potential mechanism of estrogen-dependent regulation of PRODH/POX-induced apoptosis. The effect of ER agonist, estradiol (**a**) and ER modulator, 2-methoxyestradiol (**b**) on PRODH/POX-dependent apoptosis in breast cancer cells. P5C—pyrroline-5-carboxylate; PRODH/POX—proline dehydrogenase/proline oxidase; ROS—reactive oxygen species; GLY—glycine; PRO—proline; PPAR γ—peroxisome proliferator activated receptor; ATP—Adenosine triphosphate; PPARγ—Peroxisome Proliferator Activated Receptor γ; AMPK—5′ adenosine monophosphate-activated protein kinase; E2—estradiol; 2-MOE—2-methoksyestradiol.

6. Effects of ER Modulators on PRODH/POX-Dependent Apoptosis

Phytoestrogens are natural compounds that are ER modulators. They resemble estrogens in their structure. Phytoestrogen's ability to binding ER induces an estrogenic response or an anti-estrogenic effect [76]. This effect depends on the concentration of the compound and the type of target tissue. Isoflavones at low concentrations have an agonist effect, and at higher concentrations, they are antagonists. Due to this feature, phytoestrogens are called selective estrogen receptor modulators (SERMs) [77]. Phytoestrogens exhibit a broad spectrum of anticancer activity. They inhibit proliferation, invasiveness and induce apoptosis of breast cancer cells. Furthermore, they modulate the activity of ROS-scavenging enzymes [78,79]. For instance, genistein is a characteristic isoflavone found in soybean and is the most abundant natural ERβ modulator. It has an affinity for both ERα and ERβ. However, it has a ninefold preferential affinity for ERβ. By regulating ERβ expression, genistein exerts anticancer effects. Numerous in vitro and in vivo studies have shown that genistein decreases cancer cell proliferation by blocking the cell cycle in the G2/M phase. Induction of apoptosis is associated with the activation of caspase-9 and downregulation of cyclin B1 [80,81].

Some studies have shown that genistein and other phytoestrogens have synergistic effects with other chemotherapeutics and enhance the efficacy of anticancer therapy. ER

modulators inhibit PI3K/Akt/mTOR pathway and NF-κB activation [82]. Furthermore, Akt and NF-κB inhibition leads to downregulation of Bcl-2 protein and upregulation of Bax protein [83,84]. The possible mechanism for inhibition of the PI3K/AKT pathway and enhancement of breast cancer cell apoptosis by ER modulators (e.g., equol, biochanin A, daidzein) could be related to PRODH/POX-dependent ROS generation. It has been found that ER modulators inhibit collagen biosynthesis (proline utilization process), making proline available for PRODH/POX-dependent functions [85–88]. Whether this is the case requires to be explored. However, some line of evidence supports such a hypothesis. In contrast to 17β-estradiol (the most active estrogen in the stimulation of the collagen biosynthesis), its metabolite, 2-methoksyestradiol, has the opposite effect (Figure 5b). It inhibits collagen biosynthesis (increasing the amount of intracellular proline, the substrate for PRODH/POX) and activates PPAR-γ (stimulating PRODH/POX) [89,90]. Furthermore, 2-methoxyestradiol inhibits HIF-1α [89]. Another correlation between estrogens, collagen, and PRODH/POX was found at the level of PPAR-γ. Activation of this transcription factor is known to upregulate PRODH/POX [91]. Telmisartan, PPAR-γ ligand was found to inhibit collagen biosynthesis in breast cancer cells [92], supporting free proline for PRODH/POX-dependent functions. It is supported by several studies of other authors [93,94].

It is generally accepted that estrogens induce collagen metabolism, while anti-estrogens evoke either stimulatory or inhibitory effects, depending on the concentration of anti-estrogen, cell type and microenvironmental conditions [95–97]. Anti-estrogen functions are of great importance in the biology of breast cancer. Our previous studies show that in estrogen-stimulated MCF-7 breast cancer cells, raloxifene at low concentrations (1 or 4 μM) evoked an antiestrogenic effect on collagen biosynthesis and prolidase activity, while an estrogenic effect on gelatinolytic activity. However, at high concentration (10 μM), raloxifene induced estrogenic effects on collagen biosynthesis and prolidase activity, while an antiestrogenic effect on gelatinolytic activity [85]. We also found that, at 10 μM, tamoxifen induced apoptosis in MCF-7 cells. Whether this effect is due to activation of PRODH/POX-dependent ROS formation requires to be explored.

However, recently we have found that, in estrogen-negative MDA-MB-231 breast cancer cells (expressing ERβ), cultured in estradiol-free medium, stimulation of PRODH/POX by troglitazone (TGZ) contributed to apoptosis [98]. The effect was not found in MCF-7 cells, independently of the presence or absence of estradiol in culture medium nor in MDA-MB-231 cells cultured in the medium with estradiol. It has been suggested that the mechanism involves upregulation of PRODH/POX expression (by TGZ) and attenuation of collagen biosynthesis (by eliminating estradiol-induced collagen biosynthesis), that facilitate proline availability for PRODH/POX-dependent apoptosis. The hypothesis was provided that TGZ together with anti-estrogen treatment could be considered as an approach to experimental pharmacotherapy of estrogen-negative breast cancers.

The above data suggest that ERs are involved in PRODH/POX-dependent apoptosis; however, the complexity of the mechanism of these processes requires further study. Nevertheless, based on the cited facts, it seems that blocking the function of both estrogen receptors promotes PRODH/POX-dependent apoptosis in breast cancer cells. The hypothetical mechanism of this process is shown in Figure 6.

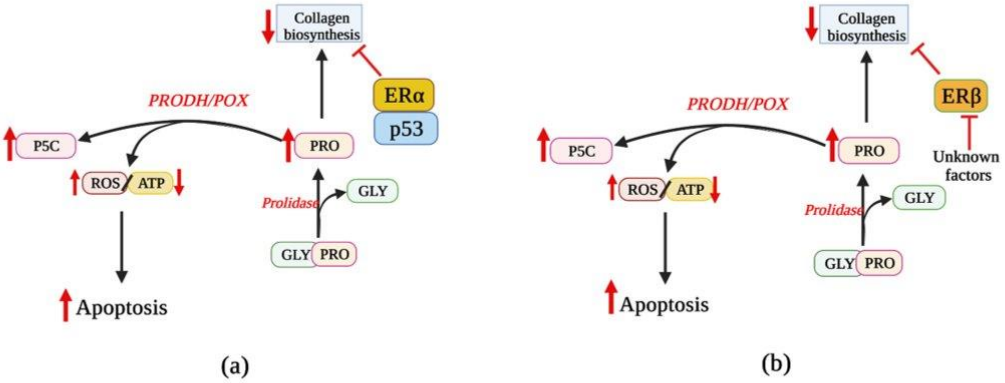

Figure 6. Downregulation of ERα and ERβ facilitates PRODH/POX-dependent apoptosis in breast cancer cells. Activation of both ERs is known to stimulate collagen biosynthesis utilizing proline as a substrate for PRODH/POX-dependent functions. (**a**) since ERα has the ability to form a complex with p53 [66], the process diminishes the potential of ERα to stimulate collagen biosynthesis contributing to an increase in proline concentration, facilitating PRODH/POX-dependent apoptosis. (**b**) The same effect could be achieved by eliminating ERβ or its inhibition by unknown factors [66,90]. P5C—pyrroline-5-carboxylate; PRODH/POX—proline dehydrogenase/proline oxidase; ROS—reactive oxygen species; GLY—glycine; PRO—proline; ATP—Adenosine triphosphate; ER—estrogen receptor.

7. Conclusions

The hypothesis that estrogens affect PRODH/POX-dependent apoptosis is based on the studies showing estrogen-induced utilization of PRODH/POX substrate (proline) for collagen biosynthesis [99,100]. In this way, estrogens stimulate proline utilization for protein synthesis, limiting its availability as a substrate for PRODH/POX-dependent apoptosis. However, estrogens may differentially affect PRODH/POX-induced functions dependently on the ERβ/ERα status. It seems that ERα has anti-apoptotic potential through antagonizing P53-dependent apoptosis, inducing the expression of HIF-1α, PPARγ, and prolidase. ERβ evokes opposite effects. The data suggest that PRODH/POX-induced apoptosis is dependent on ER status in breast cancer cells.

Therefore, in further studies on antiestrogen therapy, PRODH/POX could be considered as a target enzyme. Since tamoxifen is the only endocrine agent with approval for prevention and treatment of ER-positive breast cancers [101], it would be reasonable to perform more clinical studies on PRODH/POX-dependent apoptosis/survival in tamoxifen-treated ER-negative breast cancers.

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PUBLICATION 2

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Troglitazone-Induced PRODH/POX-Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells

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Simple Summary: PRODH/POX (proline dehydrogenase/proline oxidase) is a mitochondrial enzyme that catalyzes proline degradation generating reactive oxygen species (ROS). Estrogens limit proline availability for PRODH/POX by stimulating collagen biosynthesis. It has been considered that estrogens determine efficiency of troglitazone (TGZ)-induced PRODH/POX-dependent apoptosis in breast cancer cells. The studies were performed in wild-type and PRODH/POX-silenced estrogen-dependent MCF-7 cells and estrogen-independent MDA-MB-231 cells. DNA and collagen biosynthesis were determined by radiometric method, ROS production was measured by fluorescence assay, protein expression was determined by Western blot and proline concentration by LC/MS analysis. We found that: i/TGZ-induced apoptosis in MDA-MB-231 occurs only in the absence of estradiol or ERβ, ii/the process is mediated by PRODH/POX, iii/and is facilitated by proline availability for PRODH/POX by TGZ-dependent inhibition of collagen biosynthesis (proline utilizing process). The data suggest that combined TGZ and anti-estrogen treatment could be considered in experimental therapy of ER negative breast cancers.

Abstract: The impact of estradiol on troglitazone (TGZ)-induced proline dehydrogenase/proline oxidase (PRODH/POX)-dependent apoptosis was studied in wild-type and PRODH/POX-silenced estrogen receptor (ER) dependent MCF-7 cells and ER-independent MDA-MB-231 cells. DNA and collagen biosynthesis were determined by radiometric method, prolidase activity evaluated by colorimetric method, ROS production was measured by fluorescence assay. Protein expression was determined by Western blot and proline concentration by LC/MS analysis. PRODH/POX degrades proline yielding reactive oxygen species (ROS). Estrogens stimulate collagen biosynthesis utilizing free proline and limiting its availability for PRODH/POX-dependent apoptosis. TGZ cytotoxicity was highly pronounced in wild-type MDA-MB-231 cells cultured in medium without estradiol or in the cells cultured in medium with estradiol but deprived of ERβ (by ICI-dependent degradation), while in PRODH/POX-silenced cells the process was not affected. The TGZ cytotoxicity was accompanied by increase in PRODH/POX expression, ROS production, expression of cleaved caspase-3, caspase-9 and PARP, inhibition of collagen biosynthesis, prolidase activity and decrease in intracellular proline concentration. The phenomena were not observed in PRODH/POX-silenced cells. The data suggest that TGZ-induced apoptosis in MDA-MB-231 cells cultured in medium without estradiol or deprived of ERβ is mediated by PRODH/POX and the process is facilitated by proline availability for PRODH/POX by TGZ-dependent inhibition of collagen biosynthesis. It suggests that combined TGZ and antiestrogen treatment could be considered in experimental therapy of estrogen receptor negative breast cancers.

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1. Introduction

Troglitazone is a member of thiazolidinediones (TZD), the new class of antidiabetic drugs [1,2] and agonist of peroxisome proliferator-activated receptor-γ (PPAR-γ) [3–5].

PPAR-γ is expressed in different types of cancer cells, including breast cancer cells. Activation of the receptor was found to attenuate cell growth and induce cell death [6]. Another PPAR-γ function is up-regulation of proline dehydrogenase/proline oxidase (PRODH/POX) expression. In the promoter sequence of the gene encoding PRODH/POX there are regions binding ligand-activated receptors, the so-called PPRE, or PPAR-γ response element [3,4].

We have come up with the assumption that the mechanism of PPAR-γ dependent apoptosis could involve PRODH/POX, a mitochondrial membrane associated enzyme that catalyzes proline degradation into Δ1-pyrroline-5-carboxylic acid (P5C). During this reaction, electrons are transferred via flavin adenine dinucleotide to cytochrome c in the respiratory chain producing ATP. However, when electrons are transferred directly to oxygen then the reactive oxygen species (ROS) are formed inducing apoptosis or autophagy [7–9]. Although the mechanism for switching from ATP to ROS production is not known, several factors have been implicated in this process [10,11]. One of them is prolidase, the enzyme supporting substrate for PRODH/POX. This enzyme catalyzes the last stage of collagen degradation, releasing proline from imidodipeptides. Proline could be also derived from amino acid metabolism, mainly glutamine, α-ketoglutarate and ornithine. However, the free proline may be reused for collagen biosynthesis limiting its availability for PRODH/POX. Therefore, collagen biosynthesis may affect PRODH/POX-dependent functions, including apoptosis. It is of interest that, in several cell types, estrogens are potent stimulators of collagen biosynthesis and cell growth [12–15].

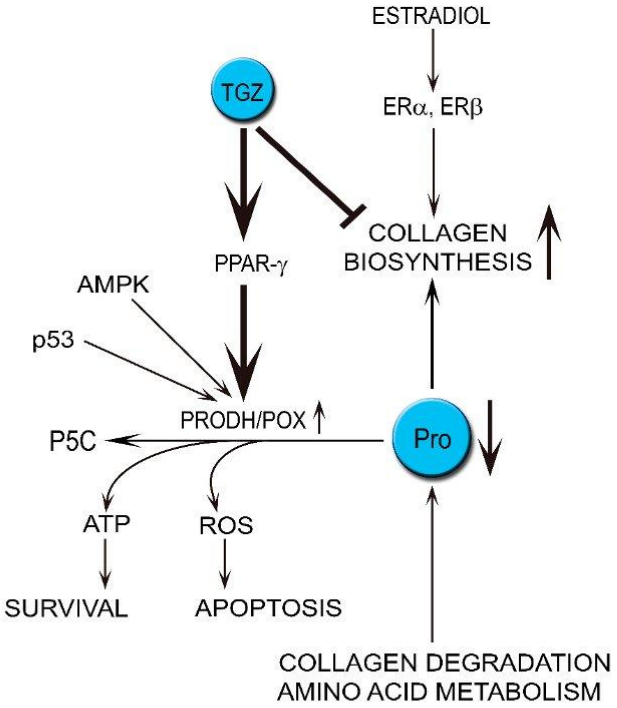
Estrogen receptor (ER) activation is considered an important factor in breast cancer progression [16]. In fact, therapeutic efficacy of antiestrogen therapy is well-established by epidemiological data [17]. Studies on estrogen receptor (ER)-positive breast cancer cell lines (e.g., MCF-7 cells expressing α and β ER) provided evidence that estrogens stimulate proliferation of the breast cancer cells both in vitro and in vivo [18,19]. However, they are poorly metastatic and more responsive to antiestrogens, compared to ER negative breast cancer cells [20]. On the other hand, ER negative breast cancer cells, (e.g., MDA-MB-231, expressing only β estrogen receptor) are highly metastatic as established in rodent models [21]. It shows that estrogens play regulatory role in breast cancer cell growth and metastasis. The mechanism of estrogen function may involve multiple factors [22]. One of them is PPAR- γ that is capable of interacting with estrogen receptors [23]. It is a family member of nuclear hormone receptors, known as a ligand-activated transcription factor [6]. Natural PPAR-γ ligands are arachidonic acid metabolites and polyunsaturated fatty acids [24]. Synthetic ligands are represented by thiazolidinediones (TZD) the new class of antidiabetic drugs, e.g., troglitazone (TGZ), rosiglitazone, pioglitazone and ciglitazone [1,2]. The agonists in association with retinoid X receptor activate PPAR-γ that binds the complex to specific recognition sites of target genes inducing their expression [3–5].

PRODH/POX is also up-regulated by AMP-activated protein kinase (AMPK) [8]. This specific protein kinase is activated by phosphorylation when the AMP/ATP ratio rises, stimulating oxidative phosphorylation to restore normal ATP levels and inhibiting energy expenditure, such as cell proliferation [25,26]. Therefore, AMPK is regulated especially in conditions of energy shortage [26] in order to inhibit anabolic processes and stimulate catabolism which characterize cancer cells. Proline derived from protein degradation serves as an energy substrate in reaction catalyzed by PRODH/POX in mitochondria [7].

As TGZ induces PRODH/POX [27], and estrogens stimulate collagen biosynthesis [12–15] utilizing free proline and limiting its availability for PRODH/POX-dependent apoptosis, it

has been considered that estrogen receptor status of breast cancer cells could determine ability of TGZ to induce PRODH/POX-dependent apoptosis.

The link between estrogens, collagen biosynthesis and degradation, PRODH/POX, PPAR-γ and apoptosis/survival led us to evaluate the impact of estrogen receptor activation on the above processes and PRODH/POX-dependent apoptosis in MCF-7 and MDA-MB-231 breast cancer cells as is outlined on the Scheme 1.



Scheme 1. The hypothesis presents the role of estradiol in stimulation of proline utilization in collagen biosynthesis, limiting proline availability for PRODH/POX-dependent functions in breast cancer cells treated with troglitazone (TGZ), ligand of PPAR-γ inducing PRODH/POX expression.

2. Materials and Methods

2.1. Cell Cultures

MCF-7 and MDA-MB-231 cells were obtained from ATCC (ATCC, Manassas, VA, USA). PRODH/POX-silenced MCF-7 and MDA-MB-231 cells were obtained as we descripted previously [28]. MCF-7 cells and MDA-MB-231 cells were maintained in DMEM without phenol red supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, and 50 µg/mL streptomycin at 37 °C in a humidified atmosphere in the presence of 5% CO_2. At about 80% of confluency the cells were treated for 24 h with estradiol (E, 2 nmol/L,), troglitazone (TGZ, 10 or 20 µmol/L) or both compounds in DMEM without phenol red supplemented with 10% CPSR1.

2.2. DNA Biosynthesis Assay

DNA biosynthesis was evaluated by [methyl-^{3}H]-thymidine incorporation into DNA as described previously [29]. The cells were cultured in 24-well plate to 80% of confluency.

After that they were incubated in medium with or without estradiol (E) and troglitazone (TGZ) for 24 h with 0.5 µCi/mL of [methyl-^{3}H]-thymidine. Incorporation of the tracer into DNA was measured by Liquid Scintillation Analyzer Tri-Carb 2810 TR and calculated using Quanto Smart TM software.

2.3. Collagen Biosynthesis

Collagen biosynthesis was evaluated by 5[^{3}H]-proline (5 µCi/mL) incorporation into proteins susceptible to bacterial collagenase. The cells were cultured in the presence of tracer in medium with or without estradiol (E) and troglitazone (TGZ) for 24 h. Incorporation of tracer into collagen was measured in accordance to the method of Peterkofsky et al. [30]. Incorporation of radioactive proline was detected by Liquid Scintillation Analyzer Tri-Carb 2810 TR and calculated using Quanto Smart TM software. Results are shown as combined values for cell plus medium fractions.

2.4. Determination of Prolidase Activity

The activity of prolidase was determined according to the method of Myara et al. [31]. The cells were cultured in 10 cm diameter dishes and incubated in medium with or without estradiol (E) and troglitazone (TGZ) for 24 h. Protein concentration was measured by the method of Lowry et al. [32]. Enzyme activity was reported as nanomoles of proline released from synthetic substrate (glycyl-proline), during 1 min per milligram of supernatant protein of cell homogenate.

2.5. Western-Immunoblot Analysis

Protein analysis was performed by Western blot as previously described [28,29]. Cell lysates of the cells were harvested and subjected to SDS-PAGE in 10% polyacrylamide gel (1 h, 125 V, room temperature (RT)). The protein was transferred to 0.22 µm pore-sized nitrocellulose (wet transfer, 1 h, 100 mA, RT). After the transfer, membranes were blocked with 5% non-fat dry milk in TBS-T (20 mmol/L Tris–HCl, 150 mmol/L NaCl, 0.05% Tween 20, pH 7.4) and incubated with rabbit anti-cleaved-caspase-9, anti-cleaved-caspase-3, anti-p53, anti-PARP, anti-cleaved-PARP, anti-AMPK alpha, anti-PPAR gamma, anti-GAPDH and goat-anti-PRODH, diluted 1:1000 in blocking buffer. Then membranes were washed in TBS with 0.05% Tween (TBST) 3 × 15 min and incubated with respective HRP-linked secondary antibody at concentration 1:7500 for 60 min at RT with gentle agitation. After washing in TBS-T (5 × 5 min) membranes were incubated with Amersham ECL Western Blotting Detection Reagent. Pictures were taken using BioSpectrum Imaging System UVP. Blots (done in three repeats) and densitometry are contained in Supplementary Data (Supplementary Figures S5–S18).

2.6. LC–MS-Based Quantitative Analysis

Proline concentration was measured according to the method of Klupczynska et al. [33]. Briefly, cells were analyzed by Agilent 1260 Infinity HPLC system coupled to Agilent 6530 Q-TOF mass spectrometry detector with electrospray ionization (Agilent Technologies, Santa Clara, CA, USA) as an ion source in positive ionization mode. Samples were injected onto a HILIC column (Luna HILIC, 2 × 100 mm, 3 µm, Phenomenex, Torrance, CA, USA) thermostated at 30 °C. Protein concentration was used to normalize the obtained results. The data was presented as a percent of the control value.

2.7. ROS Generation Assessment

Intracellular reactive oxygen species accumulation was measured using DCFH-DA as a fluorescent probe. Briefly, cells were pre-incubated with DCFH-DA (20 µM) in culture medium for 30 min, washed twice with PBS and treated with increasing concentrations of for 24 h with estradiol (E, 2 nmol/L,), troglitazone (TGZ, 10 or 20 µmol/L) or both compounds in DMEM without phenol red. The fluorescent intensity was measured at exci-

tation/emission wavelength of 488/535 nm using TECAN Infinite® M200 PRO (Männedorf, Switzerland). The results were presented as a percent of the control value.

2.8. Statistical Analysis

In the experiments presented in Figures 1 and 2, the mean values for six assays ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Shapiro–Wilk test and Kolmogorov–Smirnov tests. All results have a normal distribution. To assess statistical significance in conducted experiments, one-way ANOVA with Dunnett's multiple comparison test with 99% confidence interval was used (GraphPad PRISM v5.0, GraphPad Software Inc., San Diego, CA, USA). Results were considered significant at $p < 0.01$ level and are denoted by an asterisk (*).

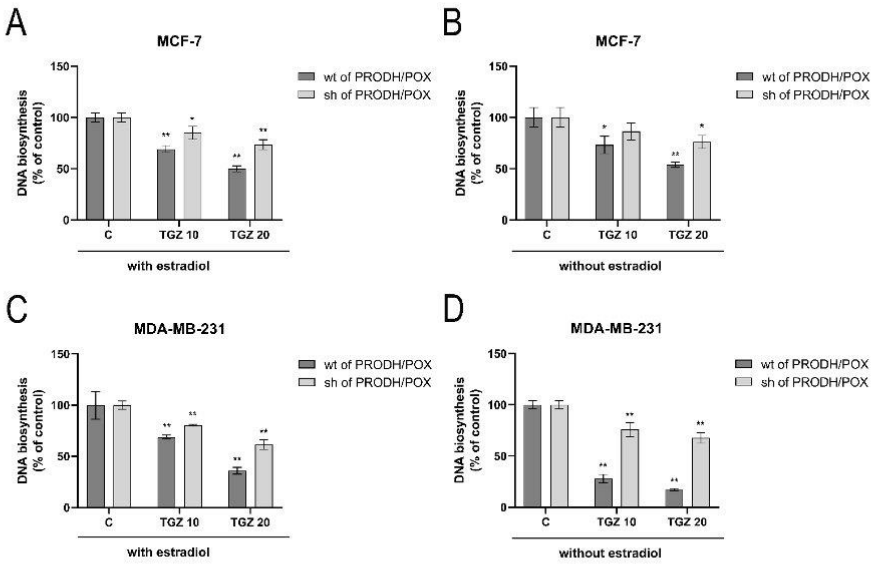


Figure 1. DNA biosynthesis in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 and MDA-MB-231 cells treated for 24 h with troglitazone (TGZ) in the presence (**A**,**C**) and absence (**B**,**D**) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks (*) indicate statistical differences between studied cells compared to controls at * $p < 0.01$ and ** $p < 0.001$.

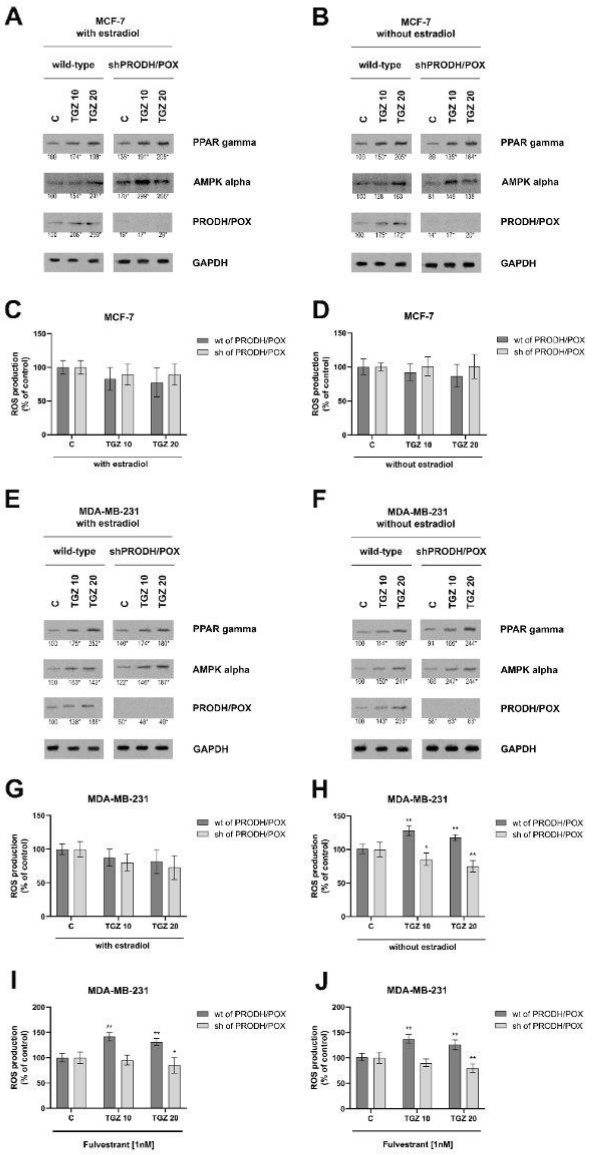


Figure 2. PRODH/POX, AMPK and PPAR-γ expressions in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 and MDA-MB-231 cells treated for 24 h with troglitazone (TGZ) in the presence (**A**,**E**) and absence (**B**,**F**) of estradiol. Representative blot images were shown (the mean value of densitometric analysis of protein bands presented below each blot;

* $p < 0.01$). Supplementary Materials contain statistical analysis of the evaluated proteins (Supplementary Material, Supplementary Figures S2–S7). ROS production is presented in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 and MDA-MB-231 cells cultured with TGZ and/or fulvestrant (ICI 182 780) in the presence (**C**,**G**,**I**) and absence (**D**,**H**,**J**) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks (*) indicate statistical differences between studied cells compared to controls at * $p < 0.01$ and ** $p < 0.001$.

3. Results

3.1. The Design of Experiments

ER-positive, MCF-7 cells (expressing α and β estrogen receptor), wild-type (wt of MCF-7 cells) and PRODH/POX-silenced (shPRODH/POX MCF-7 cells), and ER-negative, MDA-MB 231 cells (expressing only β estrogen receptor), wild-type (wt of MDA-MB-231 cells) and PRODH/POX-silenced (shPRODH/POX MDA-MB-231 cells), were used to study the effect of estrogen receptor activation on the PRODH/POX-dependent apoptosis and related processes. Preparation of PRODH/POX-silenced MCF-7 and MDA-MB-231 cells were described previously [28,29]. The representative blots with efficiency of stably silenced PRODH/POX are presented in Supplementary Materials (Supplementary Figure S1). PRODH/POX-dependent functions play critical roles in proline availability that is regulated by prolidase activity (proline supporting enzyme) and collagen biosynthesis (proline utilizing process). These processes are estrogen-dependent [12–15,34]. We used medium without phenol red, containing 10% CPSR1 and studied the effect of 2 nM estradiol on DNA biosynthesis, ROS production, proline concentration, collagen biosynthesis, prolidase activity and expression of apoptosis markers. As an inductor of PRODH/POX expression we used troglitazone (TGZ), at final concentrations of 10 and 20 μM, as previously established [34].

3.2. PRODH/POX and β-Estrogen Receptor (ERβ) Participate in TGZ-Dependent Inhibition of DNA Biosynthesis in MCF-7 and MDA-MB-231 Cells

TGZ inhibits DNA biosynthesis in both MCF-7 (Figure 1A,B) and MDA-MB-231 (Figure 1C,D) cells were cultured in the presence (Figure 1A,C) and absence (Figure 1B,D) of estradiol. The inhibition was partially dependent on PRODH/POX, as in the PRODH/POX-silenced cells the process was much less pronounced. However, in wild type MDA-MB-231 cells cultured in medium without estradiol, TGZ strongly inhibited DNA biosynthesis, while in PRODH/POX silenced cells the process was much less affected. It suggests that PRODH/POX and/or β-estrogen receptor (ERβ), that is expressed in MDA-MB-231 cells may participate in TGZ-dependent inhibition of DNA biosynthesis in these cells.

3.3. PRODH/POX and β-Estrogen Receptor (ERβ) Are Involved in TGZ-Dependent ROS Production in Breast Cancer Cells

TGZ induces PRODH/POX expression in both wild type breast cancer cells independently of the presence or absence of estradiol (Figure 2A,B). In PRODH/POX silenced breast cancer cells the enzyme was not detected and TGZ did not affect its expression. However, TGZ in a dose dependent manner induced expression of PPAR-γ and AMPK α in wild type and PRODH/POX-silenced MCF-7 as well as MDA-MB-231 cells cultured in the presence or absence of estradiol (Figure 2A,B,E,F).

TGZ did not significantly affect ROS production in both lines of MCF-7 cells cultured in the presence or absence of estradiol (Figure 2C,D) and in wild type MDA-MB-231 cells cultured in medium with estradiol (Figure 2G). However, in PRODH/POX-silenced MDA-MB-231 cells, ROS production was significantly decreased independently of the presence or absence of estradiol (Figure 2G,H). It suggests that PRODH/POX is involved in ROS production in PRODH/POX expressing breast cancer cells. Interestingly, in wild type of MDA-MB-231 cells cultured in medium without estradiol ROS production was significantly increased (Figure 2H). As MDA-MB-231 cells express only ERβ it could suggest that ERβ

may participate in TGZ-dependent generation of ROS in wild type MDA-MB-231 cells cultured in medium without estradiol. However, when ERβ was removed from MDA-MB-231 cells by ICI-182-780 (fulvestrant)-dependent degradation, TGZ regardless on the absence or presence of estradiol in the medium also induced ROS production, suggesting that ERβ is not required for the process (Figure 2I,J).

3.4. TGZ-Dependent Apoptosis in Breast Cancer Cells Is More Pronounced in Wild Type MDA-MB-231 Cells Cultured in Estradiol Free Medium than in MCF-7 Cells Cultured in the Same Conditions

TGZ induced expression of cleaved caspase-3, caspase-9 and PARP in both cell lines (MCF-7 and MDA-MB-231) cultured in the presence or absence of estradiol (Figure 3A,B; Figure 4A,B). However, their expressions were more pronounced in wild type of MDA-MB-231 cells (Figure 4B) cultured in estradiol free medium, compared to the cells cultured in medium with estradiol. Increase in the expression of studied caspases in TGZ-treated cells was accompanied by increase in the expression of p53 particularly in MDA-MB-231 cells (Figure 4B) cultured in estradiol free medium.

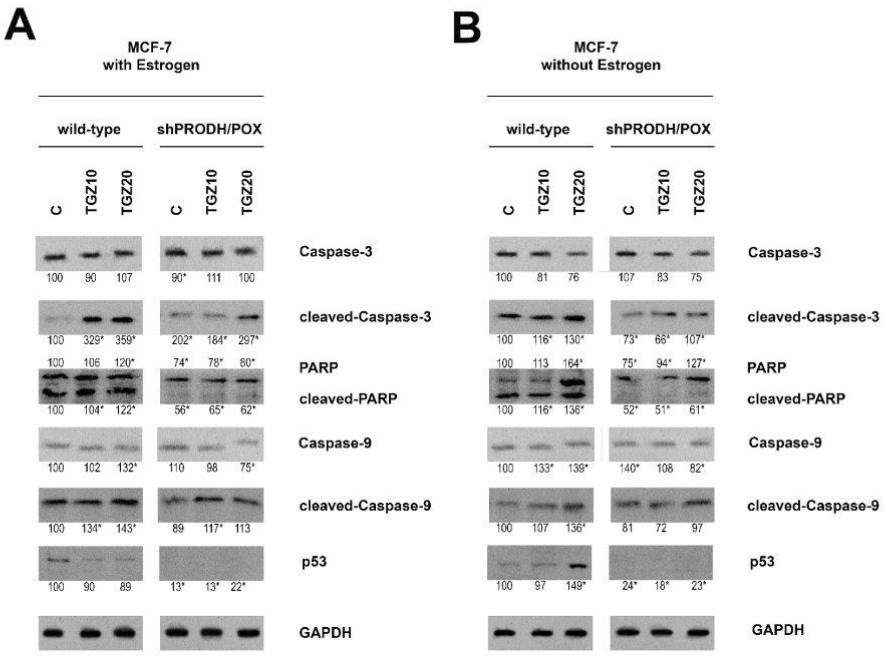


Figure 3. Western blot for cleaved caspase-3, PARP, caspase-9 and p53 in wild type and PRODH/POX-silenced (sh-PRODH/POX) MCF-7 cells treated with troglitazone (TGZ) for 24 h in the presence (**A**) and absence (**B**) of estradiol. GAPDH expression was used as a loading control. Representative blot images were shown (the mean value of densitometric analysis of protein bands presented below each blot; * $p < 0.01$). Supplementary Materials contain statistical analysis of the evaluated proteins (Supplementary Material, Supplementary Figures S8–S13).

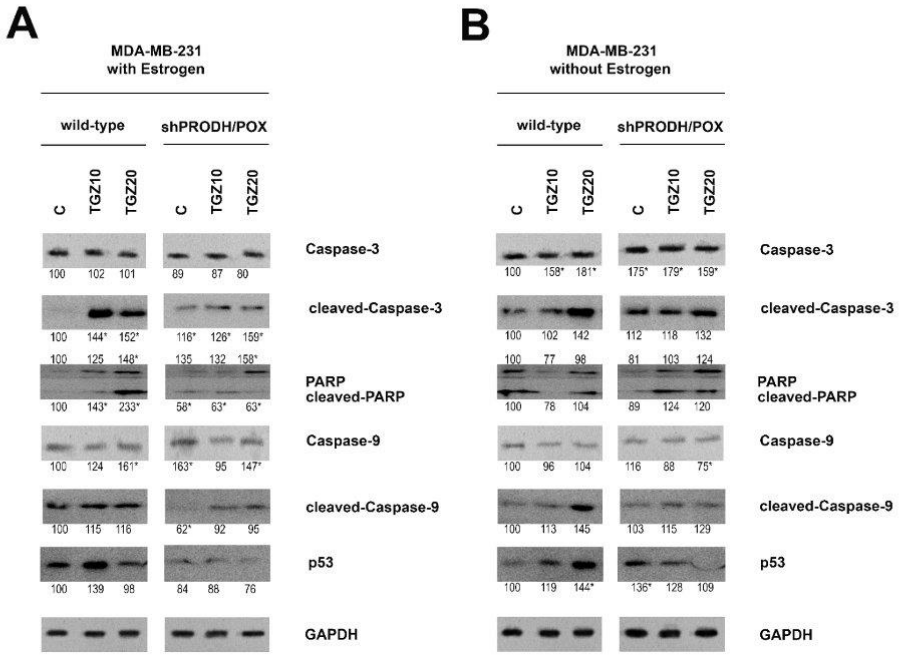


Figure 4. Western blot for cleaved caspase-3, PARP, caspase-9 and p53 in wild type and PRODH/POX-silenced (sh-PRODH/POX) MDA-MB-231 cells treated with troglitazone (TGZ) for 24 h in the presence (**A**) and absence (**B**) of estradiol. GAPDH expression was used as a loading control. Representative blot images were shown (the mean value of densitometric analysis of protein bands presented below each blot; * $p < 0.01$). Supplementary Materials contain statistical analysis of the evaluated proteins (Supplementary Material, Supplementary Figures S14–S19).

It suggests that TGZ-dependent apoptosis in breast cancer cells is highly pronounced in ERβ expressing MDA-MB-231 cells cultured in estradiol free medium.

3.5. TGZ Contributes to the Increase in Proline Availability for PRODH/POX via down Regulation of Collagen Biosynthesis and Up-Regulation of Prolidase Activity in Breast Cancer Cells

PRODH/POX-induced apoptosis (through ROS generation) in breast cancer cells is dependent on proline availability. Intracellular free proline content is regulated mainly by collagen biosynthesis (proline utilizing process) and prolidase activity (proline releasing enzyme). In wild type and PRODH/POX-silenced MCF-7 cells cultured in medium containing estradiol, TGZ induced dose-dependent increase in proline concentration (Figure 5A) and inhibition of collagen biosynthesis (Figure 5C) and prolidase activity (Figure 5E). TGZ-dependent increase in proline concentration and decrease in collagen biosynthesis and prolidase activity were more pronounced in PRODH/POX silenced cells. In MCF-7 cells cultured in medium without estradiol TGZ contributed to increase in proline concentration and decrease in collagen biosynthesis in PRODH/POX-silenced cells while in wild type MCF-7 cells the processes were much less affected (Figure 5B,D). However, TGZ inhibited prolidase activity in wild type MCF-7 cells, while it had no significant effect on the enzyme activity in PRODH/POX silenced cells cultured in estradiol free medium (Figure 5F).

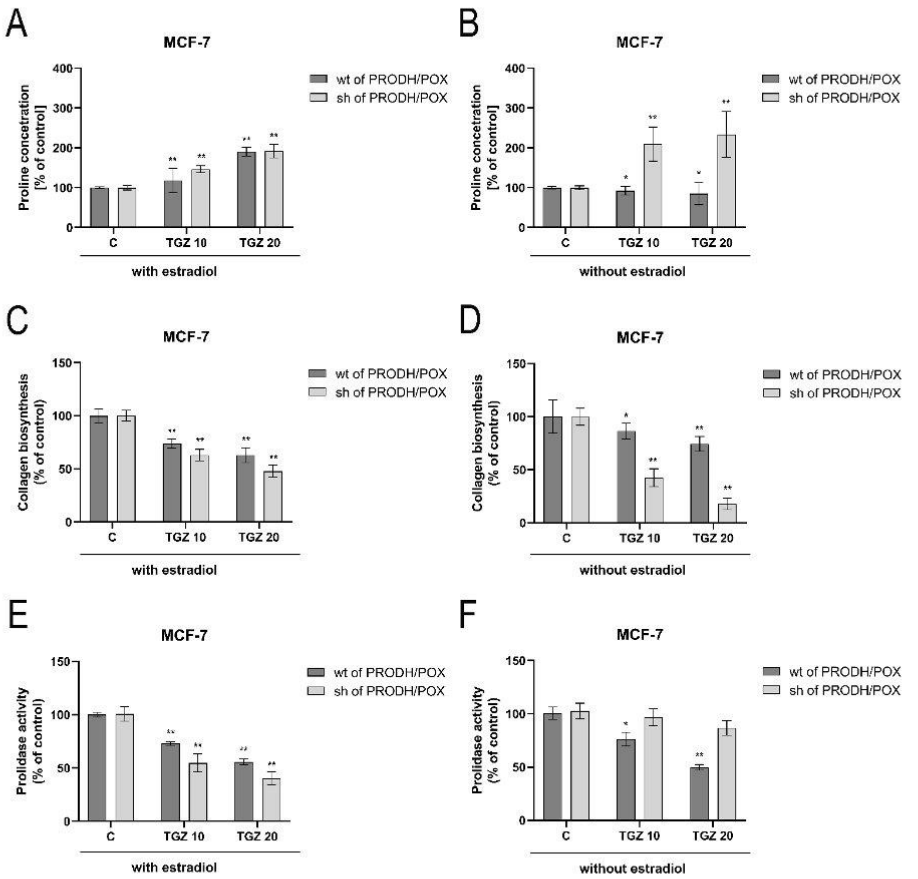


Figure 5. Proline concentration (**A**,**B**), collagen biosynthesis (**C**,**D**) and prolidase activity (**E**,**F**) in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 cells stimulated by troglitazone (TGZ) for 24 h in the presence (**A**,**C**,**E**) and absence (**B**,**D**,**F**) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks (*) indicate statistical differences between studied cells compared to controls at * $p < 0.01$ and ** $p < 0.001$.

In both wild type and PRODH/POX-silenced MDA-MB-231 cells cultured in medium containing estradiol, TGZ contributed to decrease in proline concentration (Figure 6A), collagen biosynthesis (Figure 6B) and prolidase activity (Figure 6C). The inhibition was less pronounced in PRODH/POX-silenced cells. In wild type MDA-MB-231 cells cultured without estradiol, TGZ contributed to dose-dependent decrease in proline concentration (Figure 6B), inhibition of collagen biosynthesis (Figure 6D) and prolidase activity (Figure 6F), while in PRODH/POX silenced cells, proline concentration and prolidase activity were not significantly affected and the inhibition of collagen biosynthesis was less pronounced than in MDA-MB-231 wild type cells (Figure 6B,D,F). It suggests that TGZ-induced inhibition of collagen biosynthesis could facilitate proline availability for PRODH/POX-dependent functions.

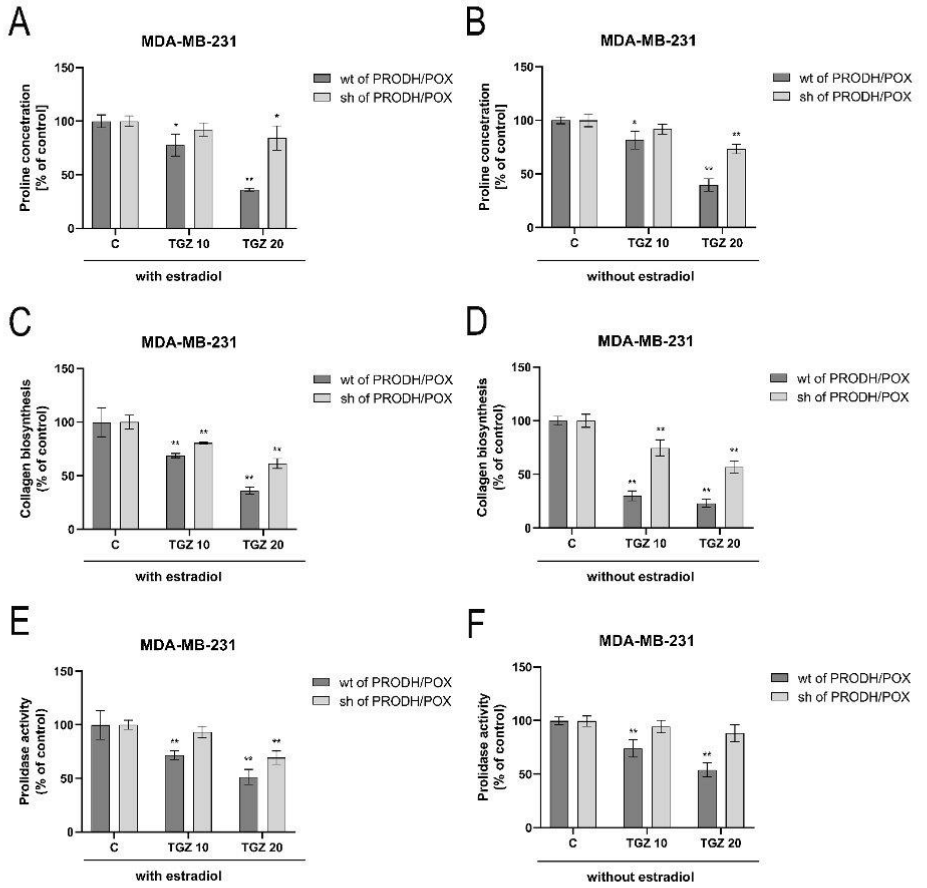


Figure 6. Proline concentration (**A**,**B**), collagen biosynthesis (**C**,**D**) and prolidase activity (**E**,**F**) in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MDA-MB-231 cells stimulated by troglitazone (TGZ) for 24 h in the presence (**A**,**C**,**E**) and absence (**B**,**D**,**F**) of estradiol. The mean values ± standard deviation (SD) from 3 experiments done in duplicates are presented. Asterisks (*) indicate statistical differences between studied cells compared to controls at * $p < 0.01$ and ** $p < 0.001$.

4. Discussion

This study investigated the role of estrogen receptor activation on troglitazone (TGZ)-induced PRODH/POX -dependent apoptosis in four models of breast cancer cells. Although cell line models have some limitations (e.g., inability to observe systemic phenomena), they are a powerful tool which offer several advantages. Certainly, the cell models allow strict control of the conditions of the experiment in order to establish the critical factor affecting the studied processes. They are especially helpful in case of limited availability of clinical samples or in vivo models (e.g., estradiol deficiency or estrogen receptor status). Therefore, results on cell models allow to predict the consequences of pharmacotherapeutic manipulation in human. Different treatment regimens and combinations of therapies have been tested using cell lines which have yielded interesting and potentially promising results that currently have an application value [35,36]. In this study we

present the result on four cell lines: estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 cell line and their respective PRODH/POX-silenced cell lines, which were described previously [28,29,37].

The data presented in this report suggest that TGZ-induced PRODH/POX-dependent apoptosis is conditioned by the status of the ERs in breast cancer cells. In MDA-MB-231 cells cultured in medium without estradiol or deprived of ERβ, TGZ induces PRODH/POX-dependent apoptosis and the process is facilitated by proline availability for PRODH/POX by TGZ-dependent inhibition of collagen biosynthesis. The underlying mechanism involves activation of PRODH/POX (by PPAR-γ ligand, TGZ) that under availability of proline (substrate for PRODH/POX) and dependently on the ER status (absence of estradiol or ERβ) induces apoptosis in ER negative breast cancer cells. The role of PRODH/POX in this process was confirmed by experiments showing that in PRODH/POX silenced cells apoptosis does not occur. The role of proline availability for PRODH/POX-dependent functions was well established [38]. Although estrogens stimulate collagen biosynthesis [12,13,15] limiting proline availability for PRODH/POX-dependent apoptosis, in the absence of estradiol and presence of TGZ the process (collagen biosynthesis) is inhibited leading to increase in the availability of substrate (proline) for PRODH/POX. We have found that in MDA-MB-231 cells cultured in medium without estradiol, TGZ contributed to apoptotic phenotype of breast cancer cells, as detected by increase in active caspase-3, -9 and PARP expressions. The effect was not found in MCF-7 cells, independently of the presence or absence of estradiol, and in MDA-MB-231 cells cultured in the medium with estradiol. The mechanism for the process was found at the level of collagen biosynthesis that is up-regulated by estrogens [27,34,39].

Collagen biosynthesis is the most effective process in utilization of intracellular proline, substrate for PRODH/POX. Previously we found that PPAR-γ ligands evoke collagen biosynthesis inhibiting activity [34]. It was proved in the present study. Such an activity of TGZ supports proline for PRODH/POX-dependent functions. However, estrogens and estrogen receptor status seem to play critical role in this process. In ER positive, wild type breast cancer MCF-7 cells treated with TGZ, the expression of PRODH/POX was increased independently of the presence or absence of estradiol, while ROS production and expression of apoptosis markers were not affected. The similar effect was found in estrogen receptor negative MDA-MB-231 cells, cultured in medium with estradiol. However, in MDA-MB-231cells, cultured in medium without estradiol, pro-apoptotic potential of TGZ was pronounced. The same effect was achieved in the cells cultured in the presence of estradiol but deprived ERβ by fulvestrant treatment. It suggests that ERβ may participate in the inhibition of PRODH/POX-dependent ROS generation in breast cancer cells. The explanation for the phenomenon is based on the fact, that estrogens activate collagen biosynthesis [13,15,34] that utilizes proline, substrate for PRODH/POX-dependent apoptosis. Removal of either ERβ or estradiol eliminate their role in stimulation of collagen biosynthesis, making proline available for PRODH/POX-dependent functions. In fact, it has been previously suggested that collagen biosynthesis is stimulated by ERβ [40] while inhibition of collagen biosynthesis induces PRODH/POX-dependent apoptosis in breast cancer cells [28].

However, in contrast to these results, it has been suggested that activation of ERβ (expressed in ER negative MDA-MB-231 cells) contributes to pro-apoptotic phenotype of breast cancer cells, while activation of ERα (expressed in ER positive MCF-7 cells) induces anti-apoptotic effects [41]. It is partially corroborated by studies showing that ER positive breast cancer cells are less metastatic, compared to ER negative ones [42,43]. In this context MDA-MB-231 cells expressing only ERβ are more invasive than MCF-7 cells, expressing both ERs. However, it is in contrast to pro-apoptotic potential of ERβ expressing cells.

It cannot be excluded that pro-apoptotic phenotype of TGZ-treated breast cancer cells, cultured in the absence of estradiol is due to other mechanisms. Some studies documented that there is a cross talk between ERs and PPAR-β [44], or ERs and P53 [41]. In such a case, estradiol could compete for binding site in ERs, therefore, the effects of ERs were seen

only in the absence of estradiol. Such a mechanism was established previously in case of ER-dependent regulation of collagen biosynthesis, where ER was removed by Fulvestrant (ICI 182-780)-induced proteasomal degradation in MDA-MB-231 cells [45]. In view of estrogen-dependent modulation of collagen biosynthesis [34,46,47] it cannot be excluded that ER/PPAR-γ cross-talk regulates proline availability for PRODH/POX-dependent functions. However, this hypothesis requires to be explored.

The absence of estradiol or ERβ in the cell culture is critical requirement for TGZ-induced PRODH/POX-dependent apoptosis in MDA-MB-231 cells. Probably, in vivo such a situation never happens, as estradiol in tissues is ubiquitous. This finding may be of importance in the experimental therapy of ER negative breast cancers by combined use of TGZ and anti-estrogens. In fact, it has been well established that estrogen receptor status determine efficacy of breast cancer therapy and its determination has predictive and prognostic value, particularly in triple-negative breast cancer [48]. However, in triple-negative breast cancer cells, MDA-MB-231, TGZ did not induce apoptosis [49]. Similarly, TGZ does not affect the viability of ER positive MCF-7 cells, however, it inhibits invasion of the cells [50]. In respect to ER positive breast cancer cells, PPAR-γ ligands as TGZ have a therapeutic limitation because it has been established that ERα blocks PPAR-γ signaling. Blocking ER by tamoxifen was shown to counteract ERα-mediated inhibition of PPAR-γ function. On the other hand, activation of ERα by estradiol blocked TGZ- induced PPAR-γ -dependent cell cycle arrest, indicating the resistance of ERα-positive breast cancer cells to TGZ. Based on these data it has been concluded that combination of troglitazone with tamoxifen may represent therapeutic approach to growth inhibition of ERα-positive MCF-7 cells [50]. It is not known whether a similar phenomenon occurs in MDA-MB-231 cells. Therefore, further studies on antiestrogen therapy accompanied by TGZ treatment should be undertaken as an approach to experimental therapy of ER negative breast cancer cells.

Downregulation of AMPK, metabolic regulator involved in control of cell growth and survival has been established as a major contributor to carcinogenesis in many types of human cancer [51]. We have found that TGZ induced in dose-dependent manner AMPK expression. Of interest is observation that TGZ induced AMPK also in PRODH/POX-silenced cells. Although AMPK is potent stimulator of PRODH/POX [27] it did not stimulate the enzyme expression in PRODH/POX silenced cells. It seems that the efficiency of this stimulation depends on constitutive level of PRODH/POX expression.

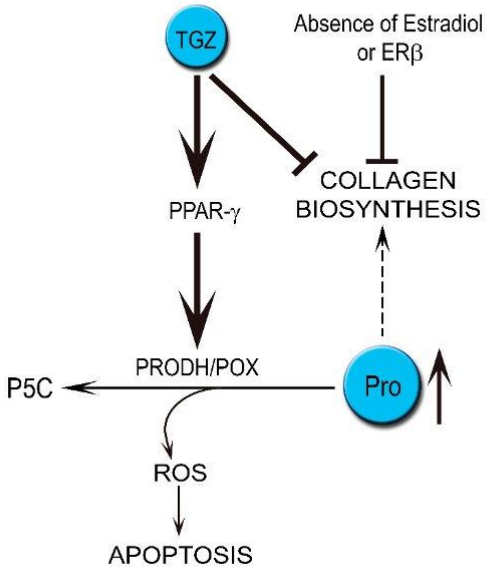
Another interesting factor in studies on PRODH/POX dependent apoptosis is p53 protein. This transcription factor is the best characterized apoptosis inducing factor and also the most potent factor regulating expression of PRODH/POX. The presence of response element for p53 protein in the promoter sequence of the gene coding PRODH/POX has been demonstrated. It shows direct participation of p53 in the transcription of PRODH/POX [11]. However, in MDA-MB-231 cells it has probably low importance due to mutation [52,53] that eliminate the transcription factor as a player in the mechanism driving PRODH/POX-dependent apoptosis in ER negative breast cancer cells. Nevertheless, p53 expression was up-regulated in TGZ-treated breast cancer cells cultured in estradiol-free medium. Interestingly, a pro-apoptotic effect of AMPK in cancer cells and a mutual relationship between AMPK and p53 have been reported. It cannot be excluded that pro-apoptotic effect of TGZ involves cross-talk between AMPK and p53 [51]. Although the mechanism is not well understood, the activation of p53 in the cells cultured in estradiol-free medium is supported by some other authors, suggesting that estradiol inactivates P53 [54] or estrogen receptor prevents p53-dependent apoptosis in breast cancer [55,56]. It seems that in the absence of estradiol, TGZ-treated MCF-7 cells may undergo apoptosis by p53 signaling [50,51], while MDA-MB-231 cells, due to p53 mutation, through PRODH/POX-dependent ROS generation.

Presently, the selective ER modulator, tamoxifen, is the only endocrine agent with approval for prevention and treatment of ER positive breast cancer [57]. In view of the presented results it would be reasonable to perform more clinical studies on tamoxifen treated ER negative breast cancer cells. Although the cells lack ERα, the expression of ERβ

is sufficient to trigger estrogen-responsivity via ERα-independent pathways [58]. It has been reported that estrogens promote the brain metastatic colonization of TNBC cells [59] while ovariectomy decreased the frequency of brain metastases as compared to estrogen supplementation, and that the combination of ovariectomy and aromatase inhibitor further reduced the frequency of large lesions to 14% of the estrogen control. Furthermore, it was demonstrated [60] that increasing levels of circulating estrogens was sufficient to promote the formation and progression of ERα-negative cancers. These data suggest that endocrine therapy options directed against ERβ and estrogens should be considered for treatment of ER negative breast cancer.

5. Conclusions

The data suggest that TGZ-induced apoptosis in MDA-MB-231 cells cultured in medium without estradiol or deprived of ERβ is mediated by PRODH/POX and the process is facilitated by proline availability for PRODH/POX by TGZ-dependent inhibition of collagen biosynthesis (Scheme 2). It suggests that combined TGZ and anti-estrogen treatment could be considered in experimental therapy of ER negative breast cancers.



Scheme 2. The role of ER status (presence or absence of estradiol or ERβ) in the mechanism of TGZ-induced PRODH/POX-dependent apoptosis in breast cancer MDA-MB-231 cells. ER—estrogen receptor; P5C—Δ1-pyrroline-5-carboxylic acid; PPAR-γ—peroxisome proliferator-activated receptor γ; Pro—proline; ROS—reactive oxygen species; TGZ—troglitazone.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10204641/s1, Figure S1: Representative blots of expression of PRODH/POX (POX) in MCF-7 and MDA-MB-231 cells; Figure S2: The PPAR expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S3. The PPAR expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S4: The AMPK expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S5: The AMPK expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S6: The PRODH/POX (POX)

expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S7: The PRODH/POX (POX) expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S8: The non-cleaved-Caspase-3 expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S9: The cleaved-Caspase-3 expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S10: The PARP (upper bands) and cleaved-PARP (lower bands) expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S11: The non-cleaved-Caspase-9 expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S12: The cleaved-Caspase-9 expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S13: The p53 expressions in MCF-7WT cells and MCF-7shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S14: The non-cleaved-Caspase-3 expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S15: The cleaved-Caspase-3 expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S16: The PARP and cleaved-PARP expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S17: The non-cleaved-Caspase-9 expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S18: The cleaved-Caspase-9 expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.; Figure S19: The cleaved-Caspase-3 expressions in MDA-MB-231WT cells and MDA-MB-231shPRODH/POX cells cultured in DMEM in the presence and absence of estradiol.

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SUPPLEMENTARY MATERIAL - PUBLICATION 2

P2. Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone- Induced PRODH/POX- Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4.242. DOI: 10.3390/jcm10204641.

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Supplementary Material

Article

Troglitazone-Induced PRODH/POX-Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells

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1. Results

1.1. *Efficacy of shRNA-based PRODH/POX knock-down in MCF-7 and MDA-MB-231 cells*

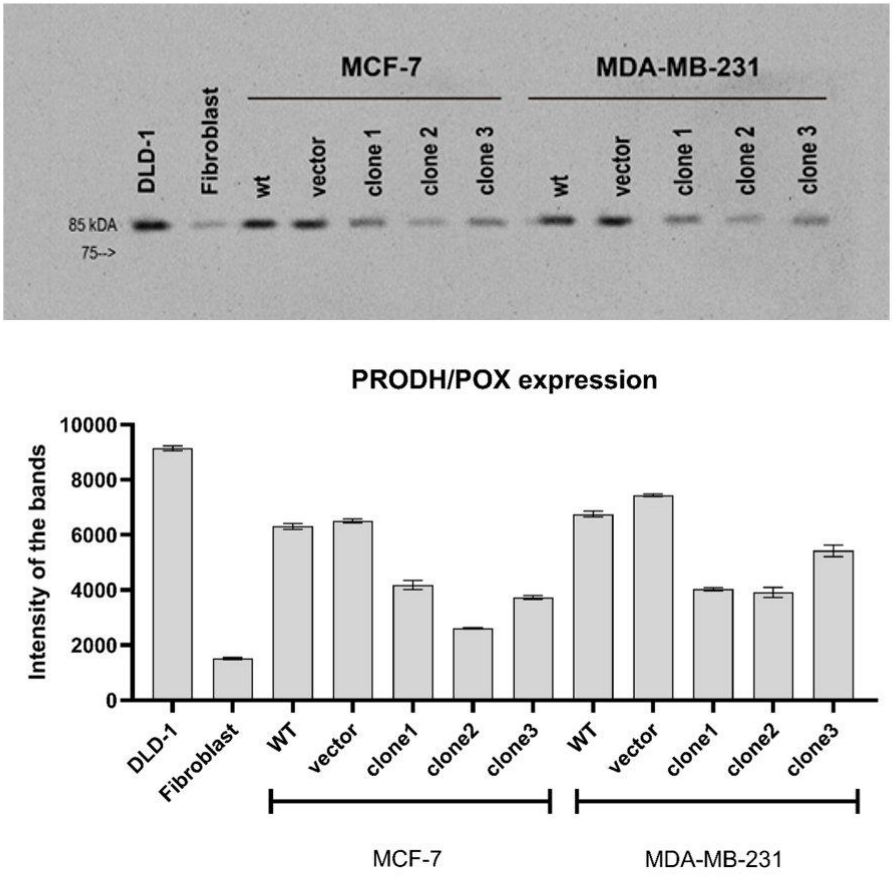
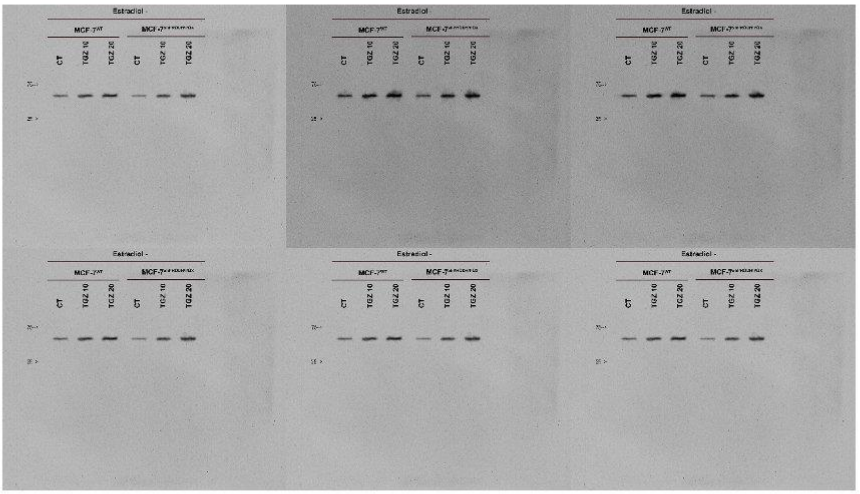


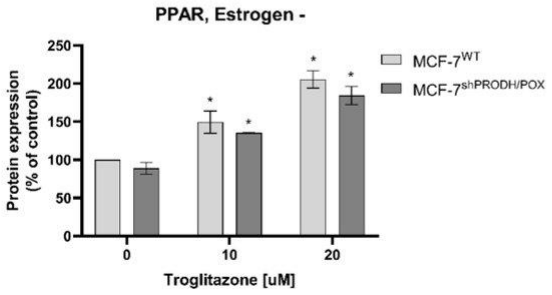
Figure S1. Representative blots of expression of PRODH/POX (POX) in MCF-7 and MDA-MB-231 cells. Transfection of the cells with different PRODH/POX shRNA constructs (clone1-3) were done. DLD-1 cells were used as a positive control and fibroblasts as a negative control for the expression of PRODH/POX. Representative gels of Western blotting and the intensity of POX bands was quantified by densitometry and normalized to GAPDH, values represent the mean (% of control) ± SD of three experiments, $*p < 0.001$.

1.2. *Western blots presented on Figure 2*

1.2.1. PPAR gamma

1.2.1.1. MCF-7 cell line





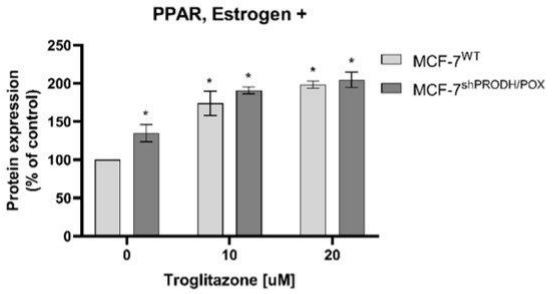
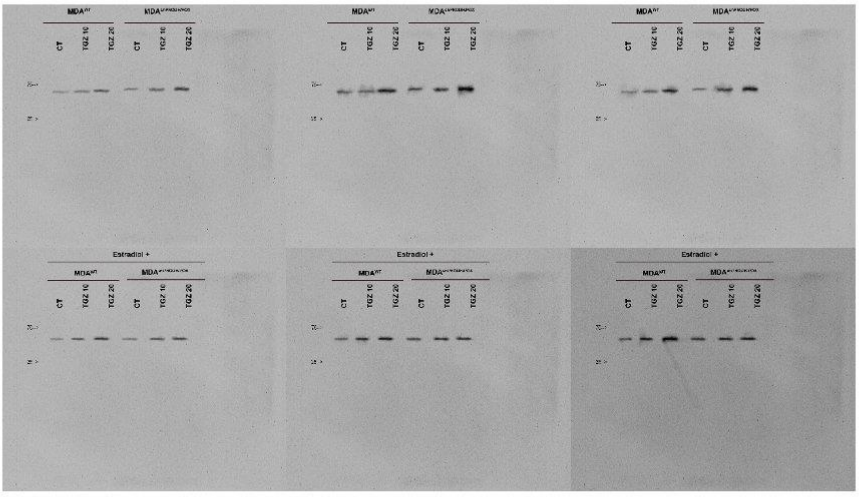


Figure S2. The PPAR expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, *$p < 0.001$.

1.2.1.2. MDA-MB-231 cell line



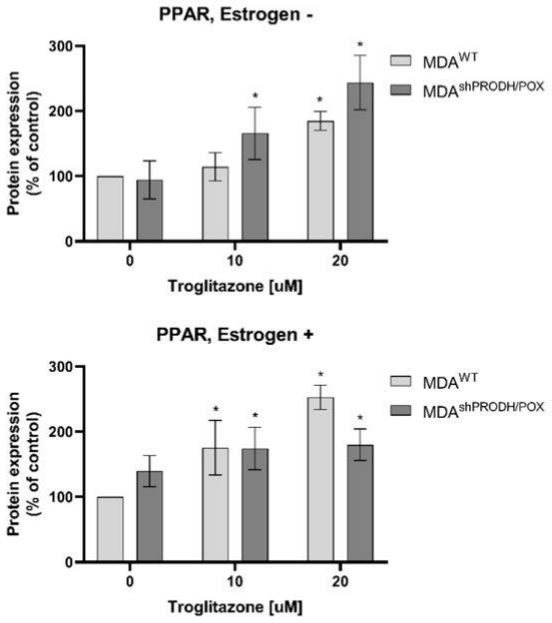
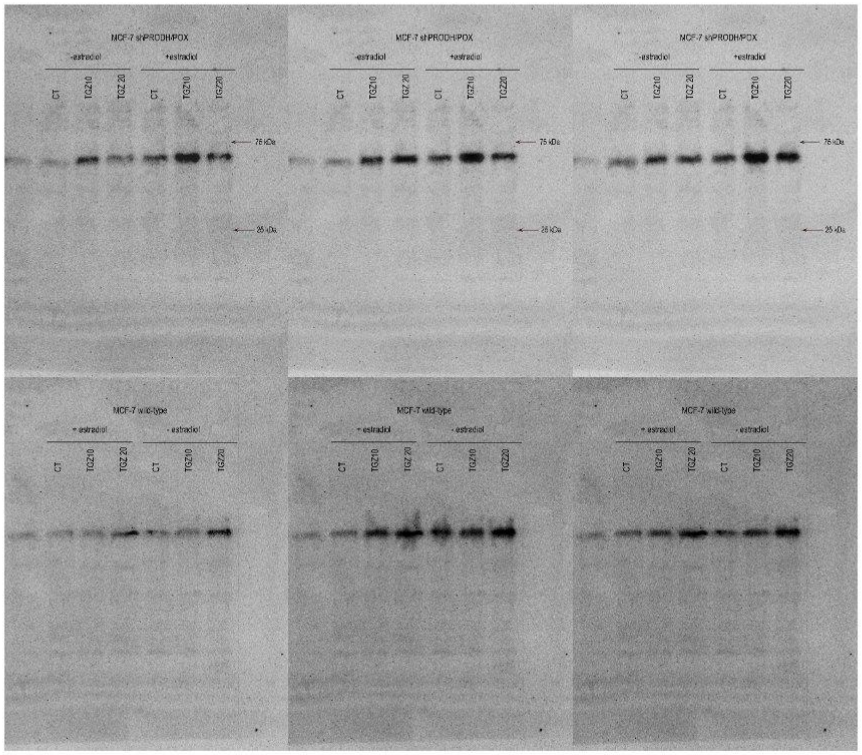


Figure S3. The PPAR expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.2.2. AMPK alpha

1.2.2.1. MCF-7 cell line



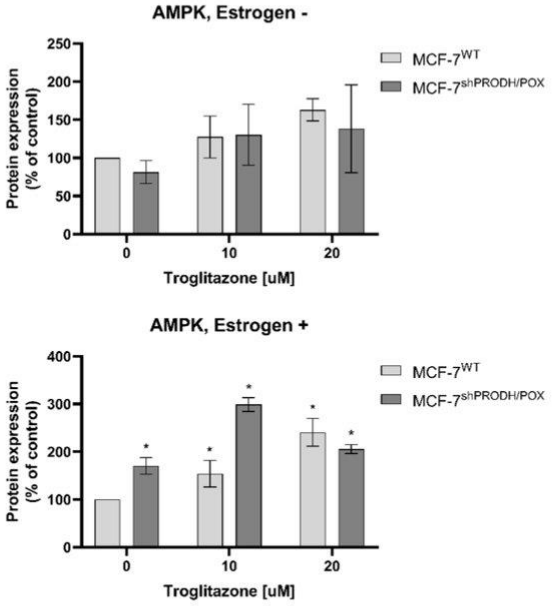
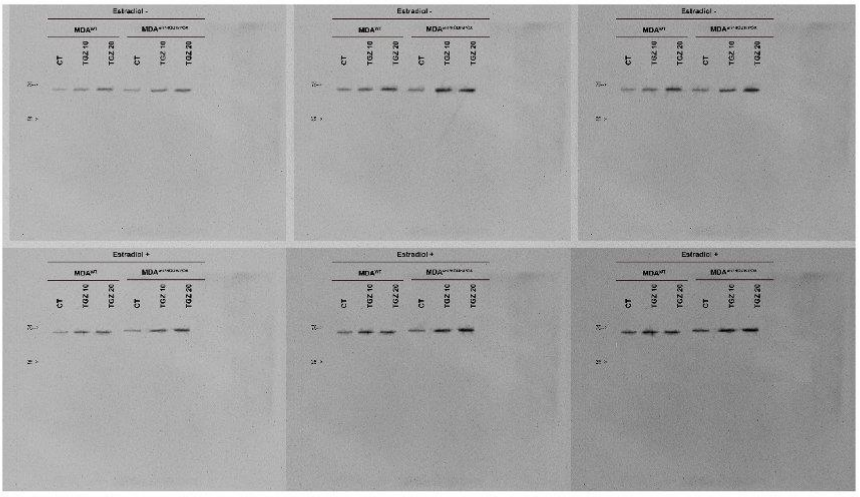


Figure S4. The AMPK expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.2.2.2. MDA-MB-231 cell line



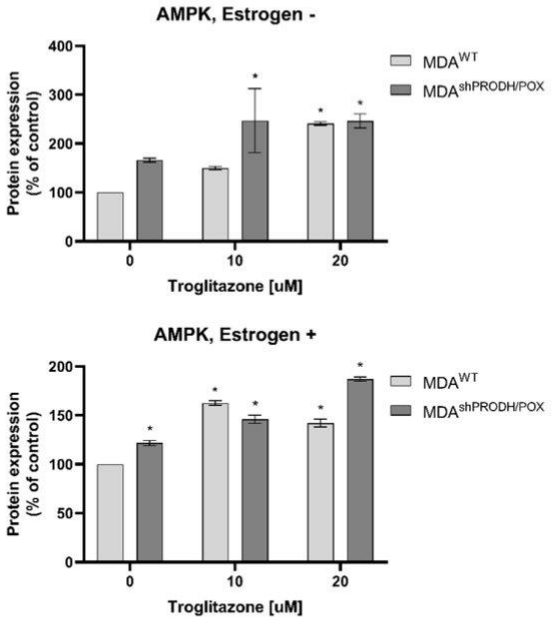


Figure S5. The AMPK expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.2.3. PRODH/POX

1.2.3.1. MCF-7 cell line

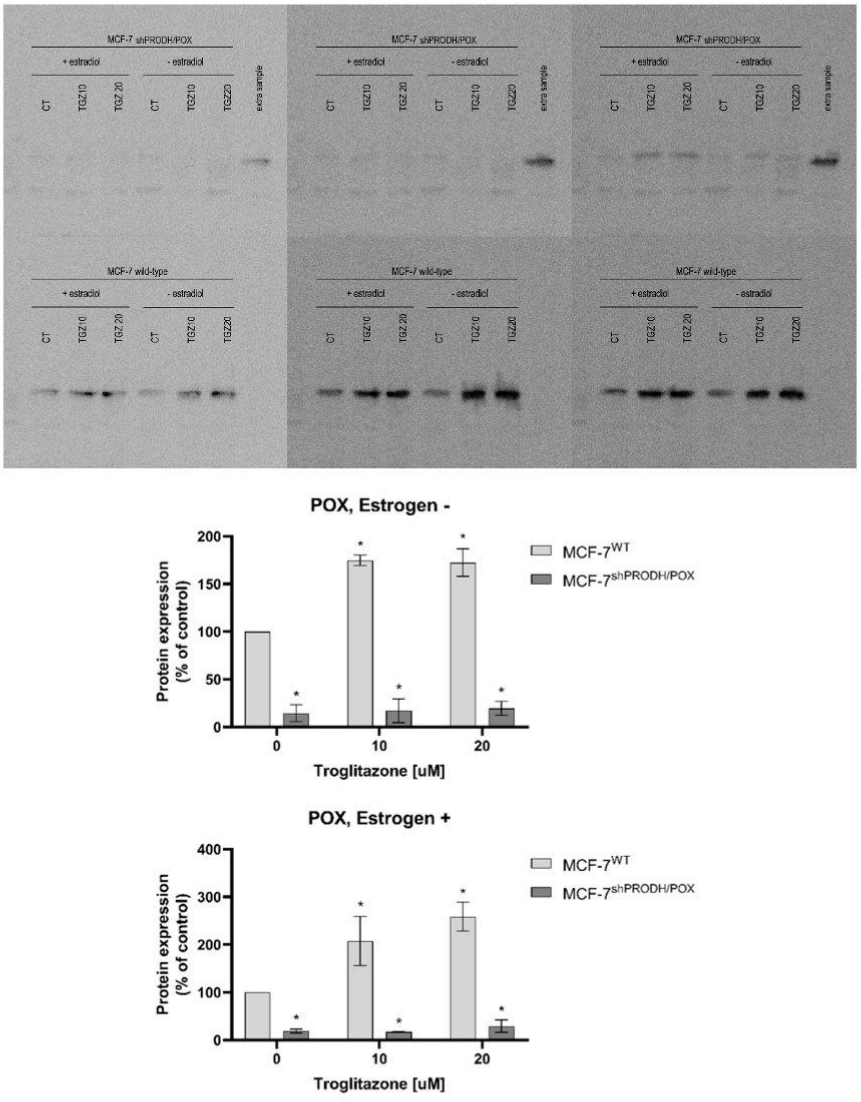
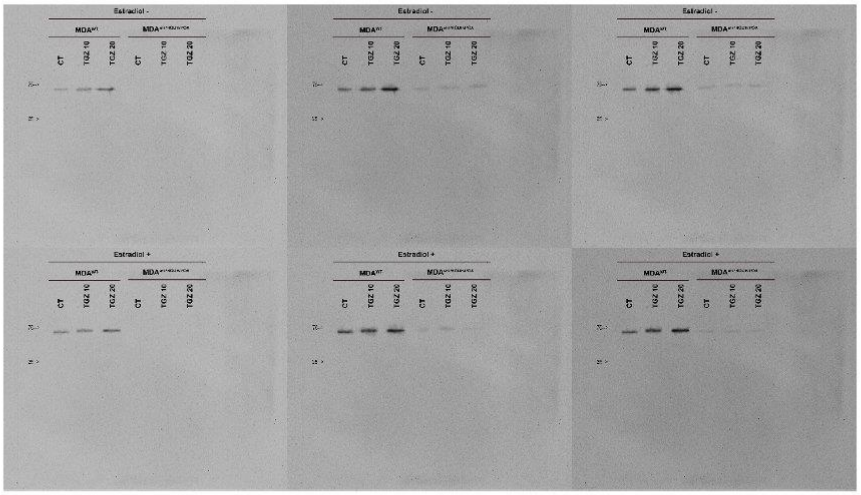


Figure S6. The PRODH/POX (POX) expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $*p < 0.001$.

1.2.3.2. MDA-MB-231 cell line



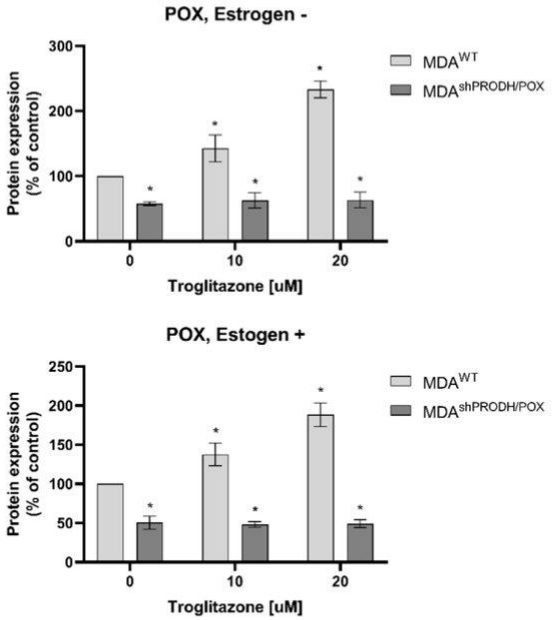
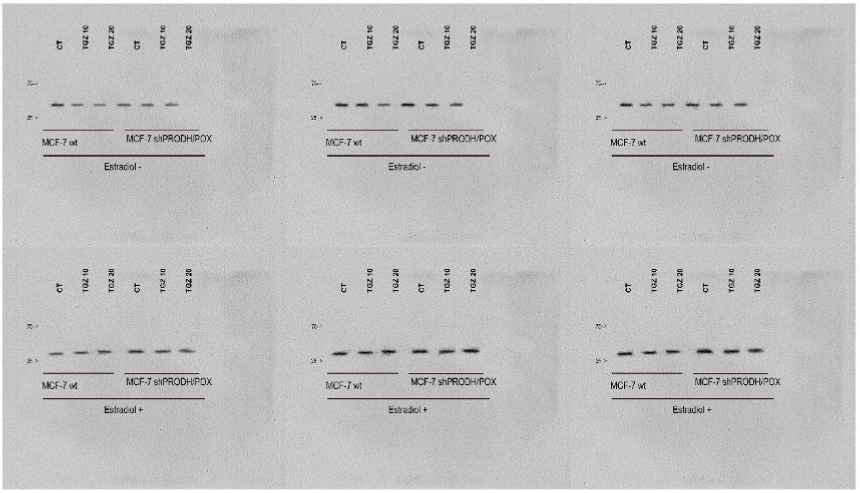
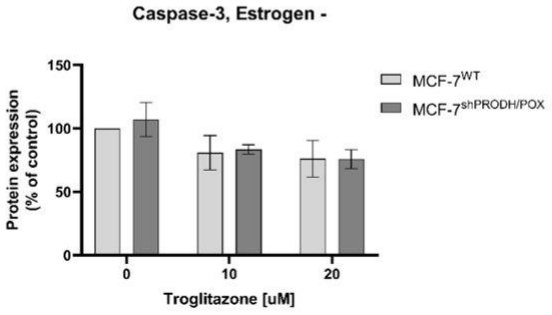


Figure S7. The PRODH/POX (POX) expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.3. *Western blots presented on Figure 3*

1.3.1. *Caspase-3*





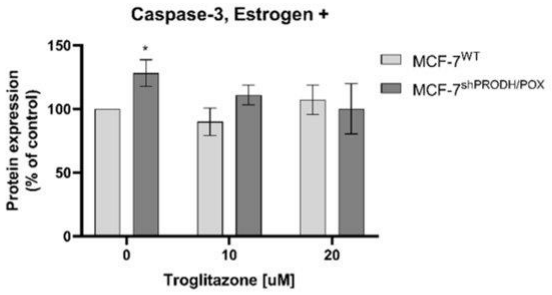
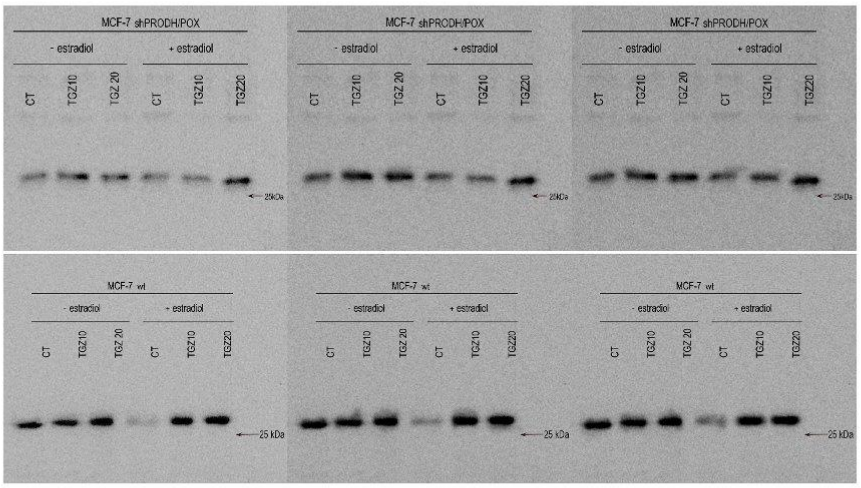
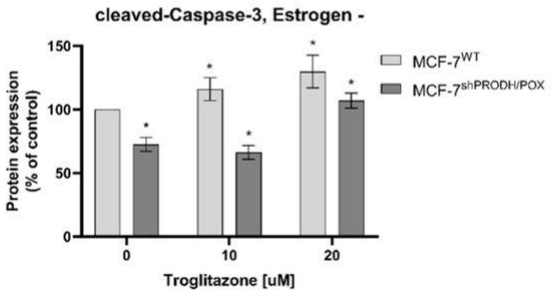


Figure S8. The non-cleaved-Caspase-3 expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.3.2. *Cleaved-Caspase-3*





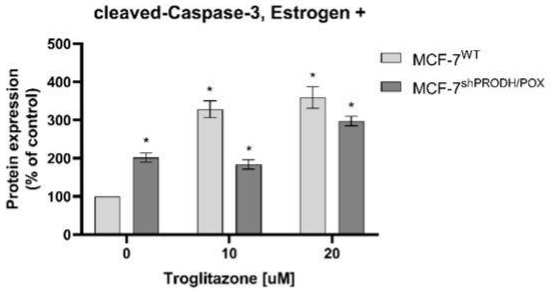
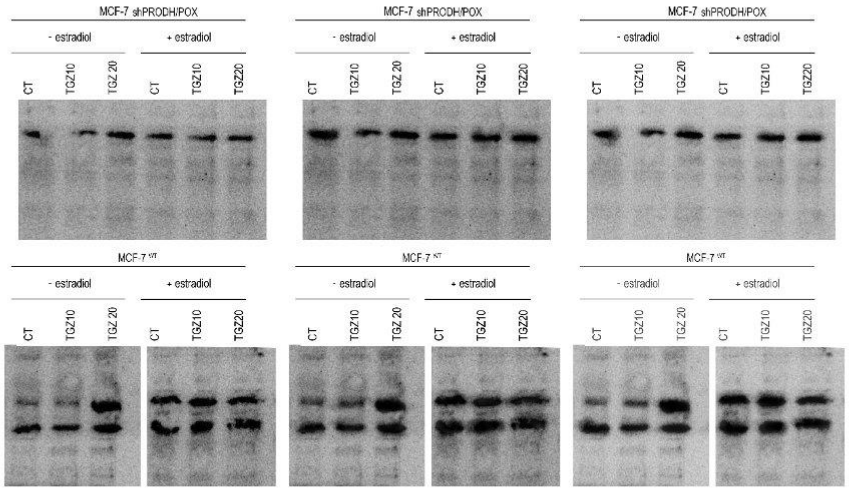
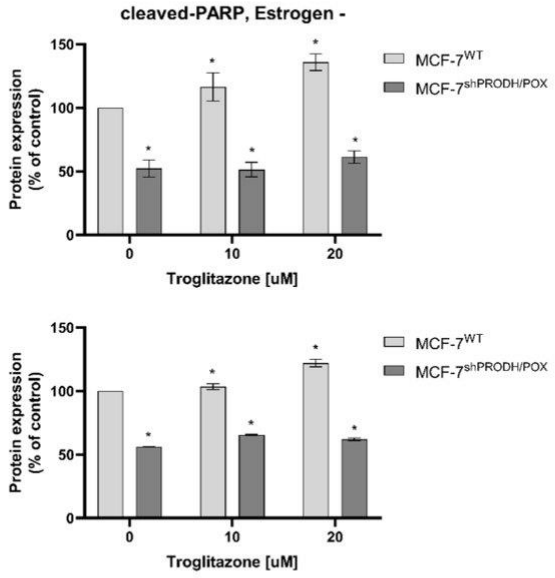


Figure S9. The cleaved-Caspase-3 expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.3.3. *PARP and cleaved-PARP*

PARP (upper bamds) and cleaved-PARP (lower bands)





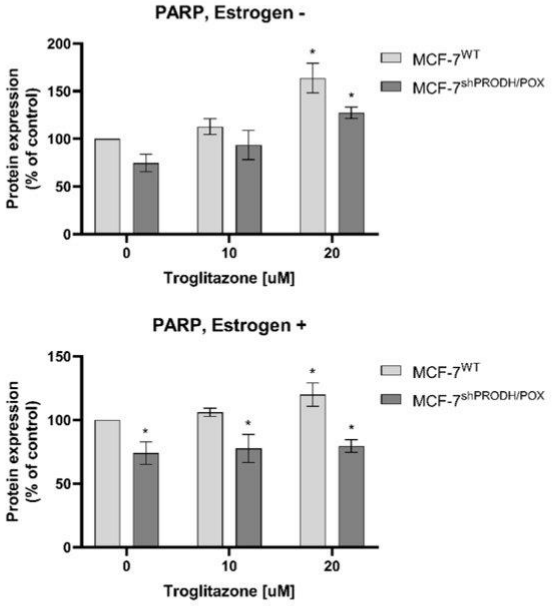
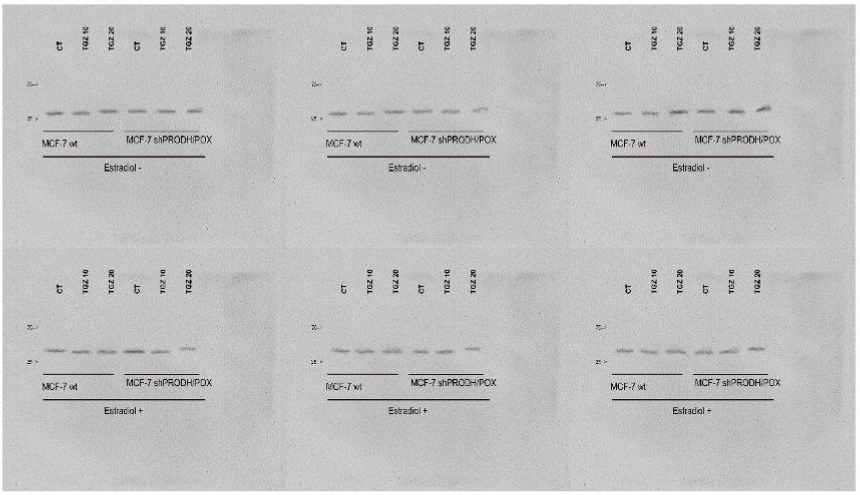


Figure S10. The PARP (upper bands) and cleaved-PARP (lower bands) expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $*p < 0.001$.

1.3.4. *Caspase-9*



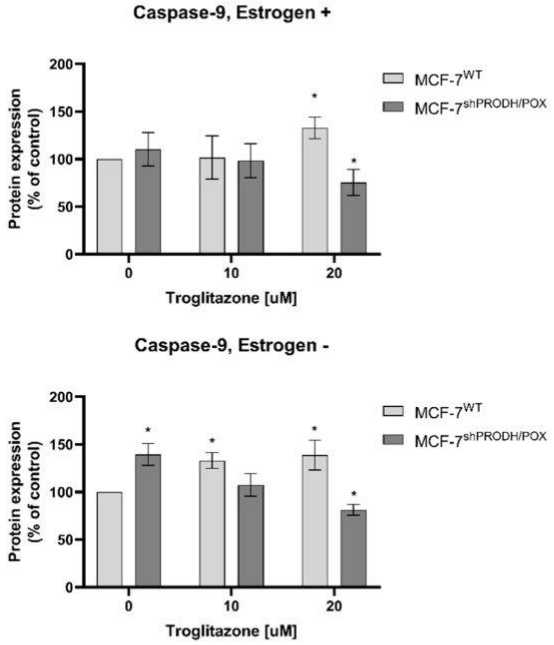
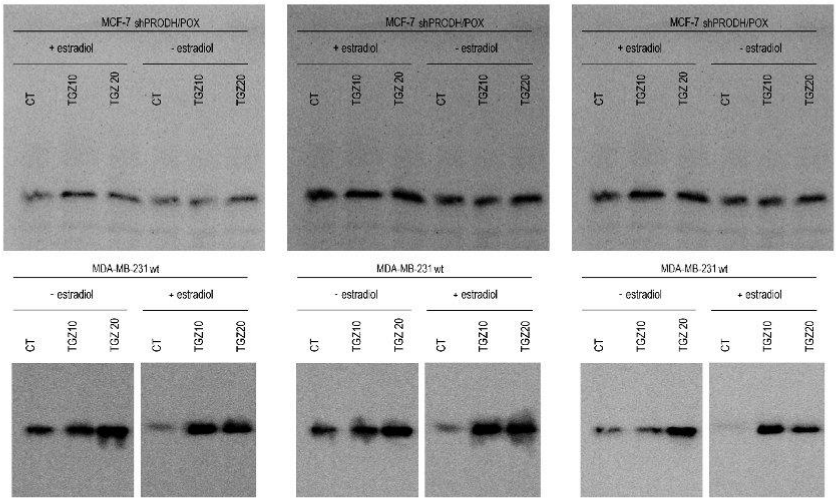
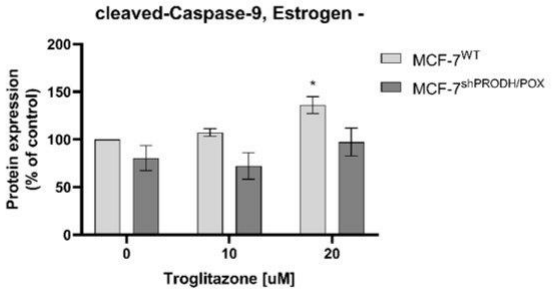


Figure S11. The non-cleaved-Caspase-9 expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.3.5. *Cleaved-Caspase-9*





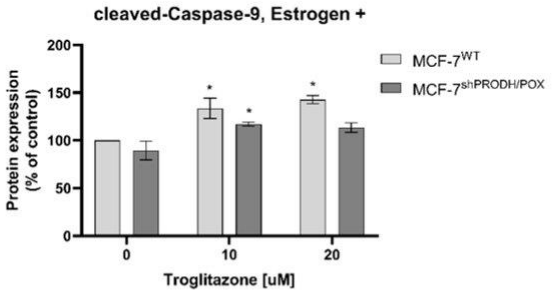
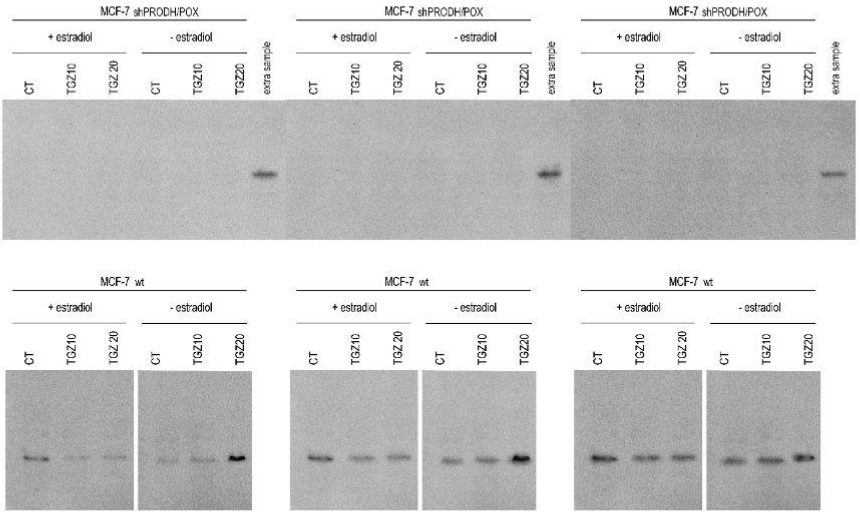
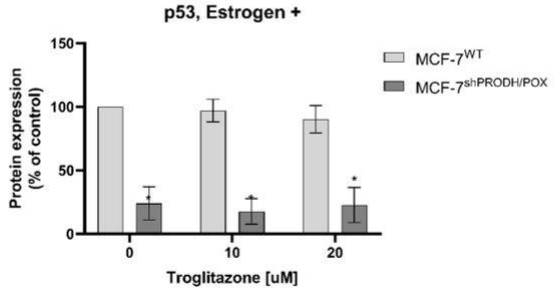


Figure S12. The cleaved-Caspase-9 expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.3.6. *p53*





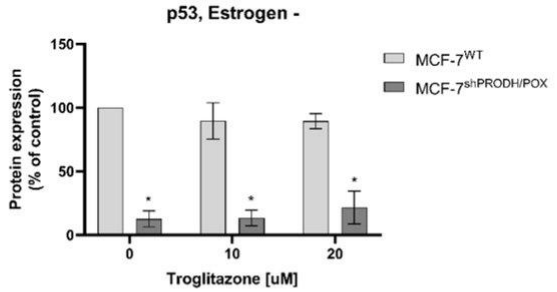
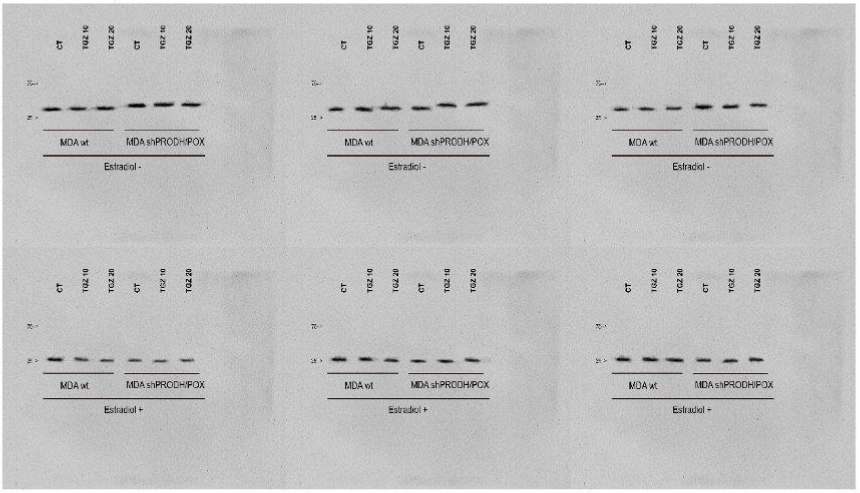


Figure S13. The p53 expressions in MCF-7^{WT} cells and MCF-7$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.4. *Western blots presented on Figure 5*

1.4.1. *Caspase-3*



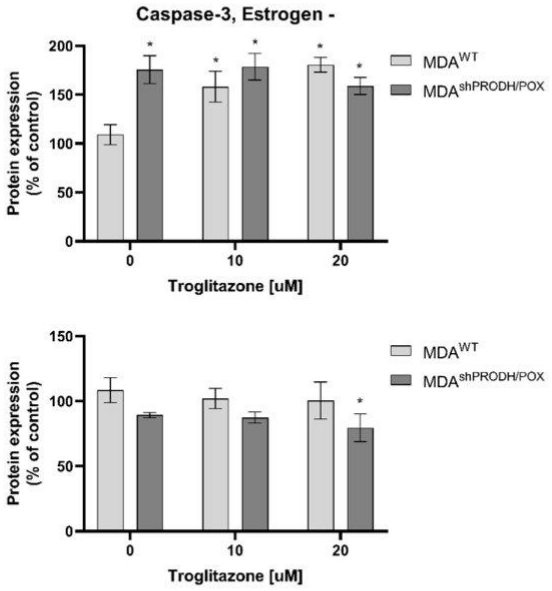
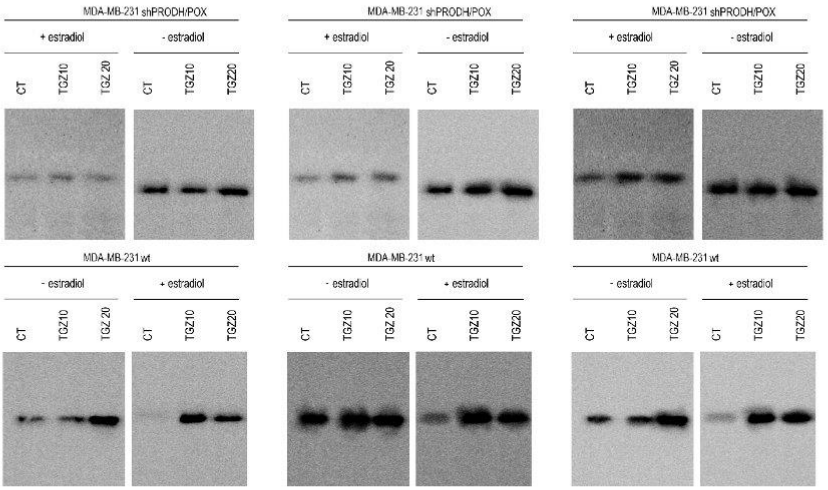


Figure S14. The non-cleaved-Caspase-3 expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $*p < 0.001$.

1.4.2. ***Cleaved-Caspase-3***



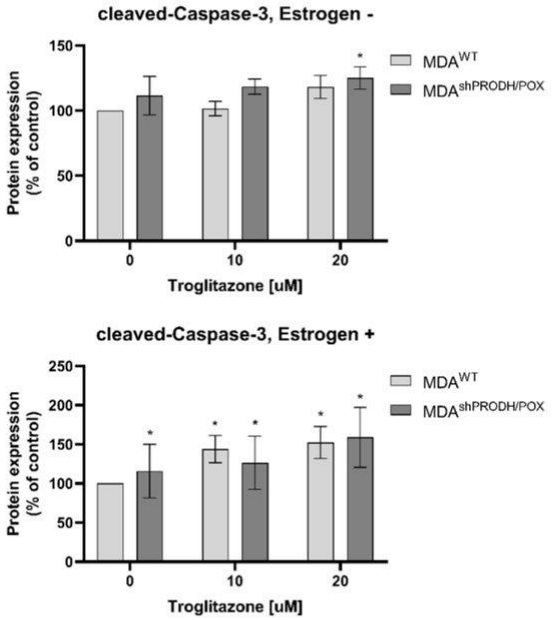
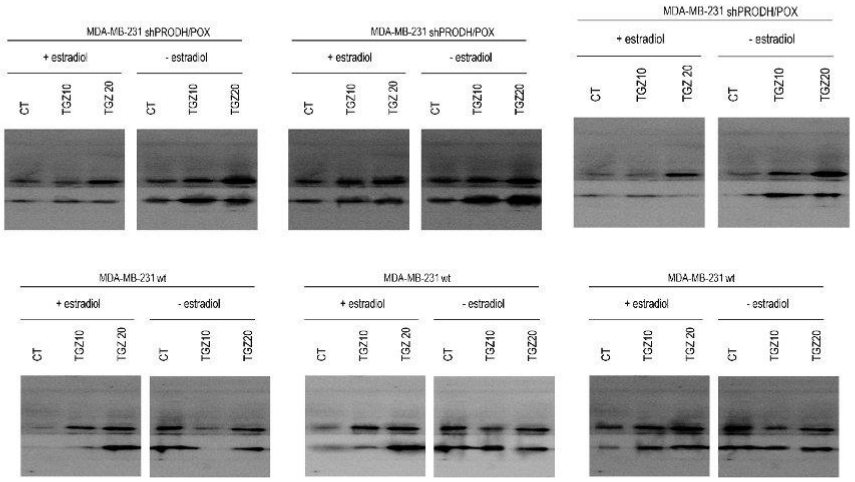
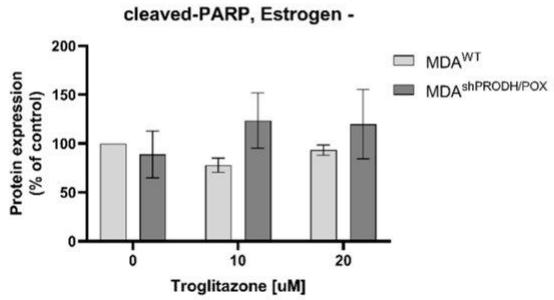


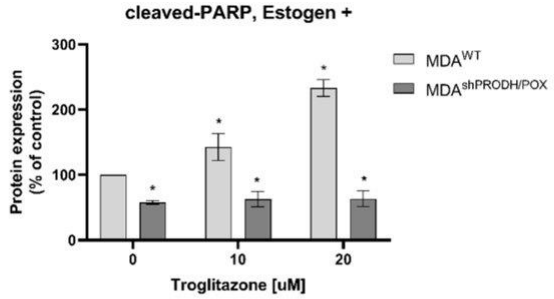
Figure S15. The cleaved-Caspase-3 expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.4.3. *PARP and cleaved-PARP*

PARP (upper bamds) and cleaved-PARP (lower bands)







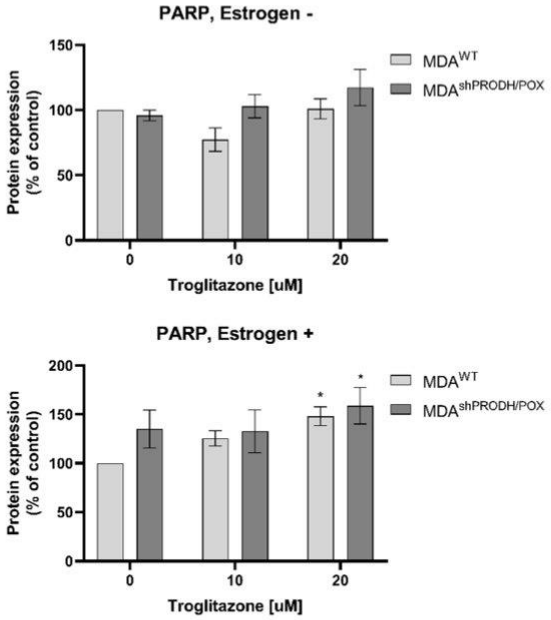
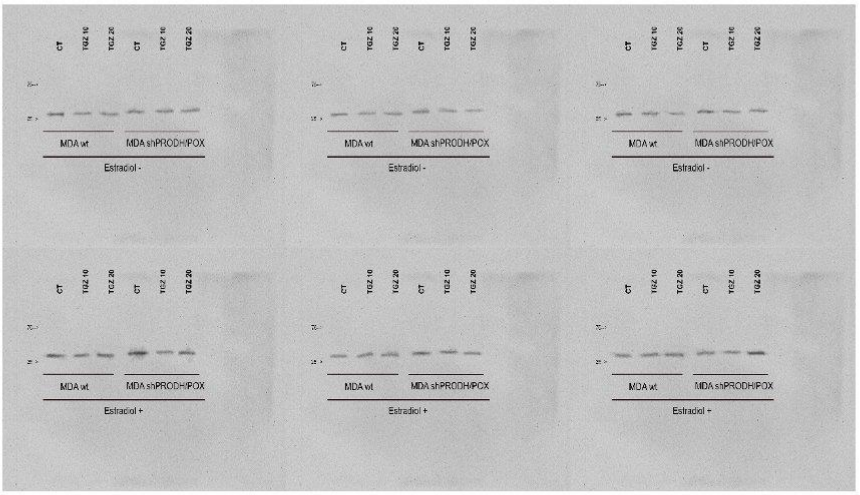
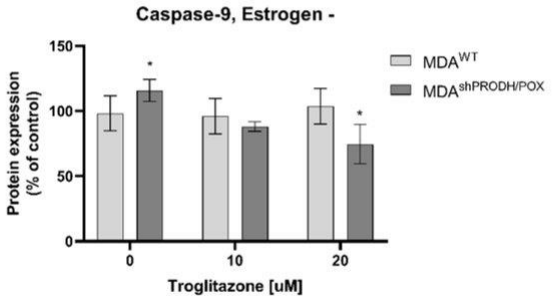


Figure S16. The PARP and cleaved-PARP expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.4.4. *Caspase-9*





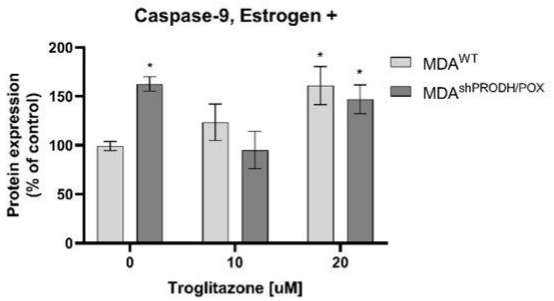
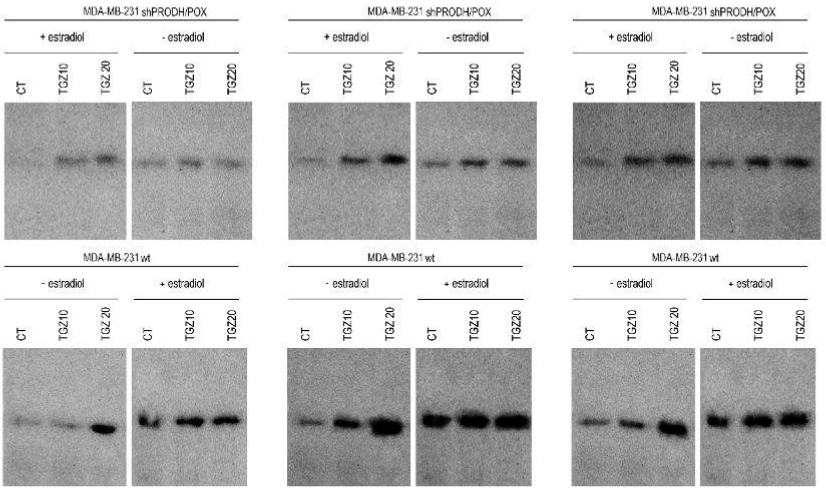


Figure S17. The non-cleaved-Caspase-9 expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $*p < 0.001$.

1.4.5. ***Cleaved-Caspase-9***



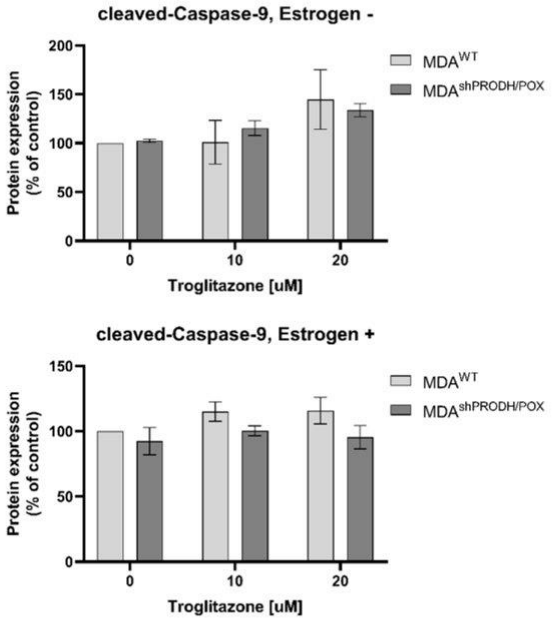
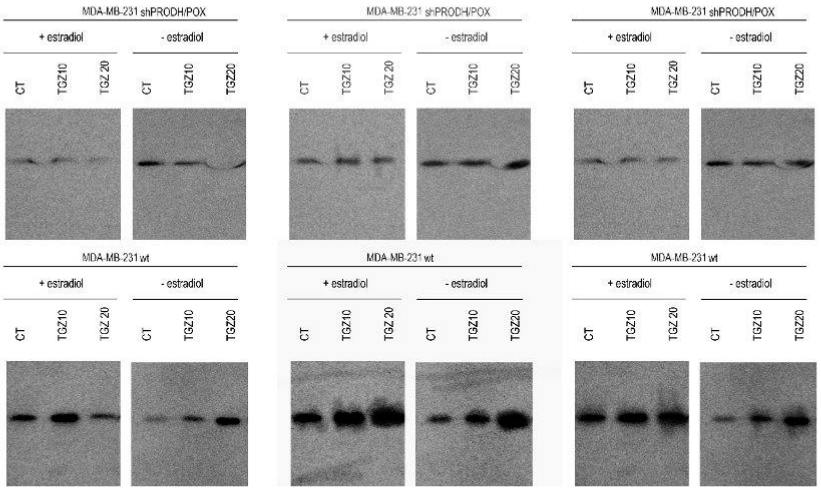


Figure S18. The cleaved-Caspase-9 expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

1.4.6. *p53*



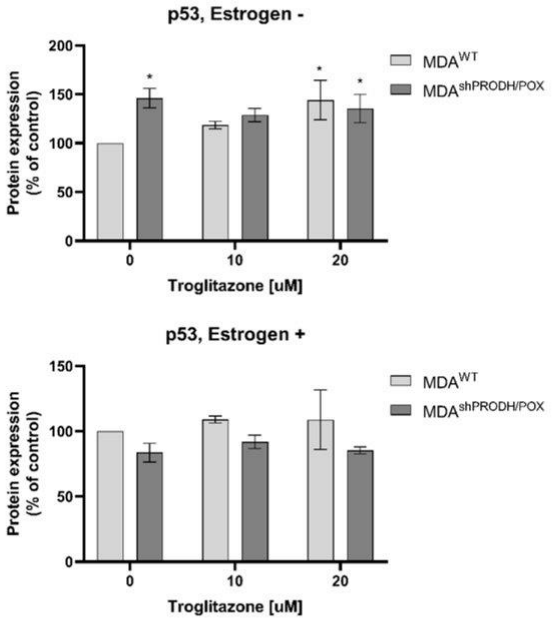


Figure S19. The cleaved-Caspase-3 expressions in MDA-MB-231^{WT} cells and MDA-MB-231$^{shPRODH/POX}$ cells cultured in DMEM in the presence and absence of estradiol. GAPDH expression was used as a loading control. The WB bands intensity of representative gels was quantified by densitometry and normalized to GAPDH. The densitometry values represent the mean (% of control) ± SD of three experiments, $^{*}p < 0.001$.

SUMMARY

This report provides evidence for the important role of estrogens in the mechanism of PRODH/POX-dependent apoptosis in breast cancer cells.

PRODH/POX is a mitochondrial enzyme catalyzing the conversion of proline to Δ1-pyrroline-5-carboxylic acid (P5C). During the conversion of proline to P5C, electrons are transported to the respiratory chain, producing ATP or they are directly accepted by oxygen, generating reactive oxygen species (ROS). In the first case, activation of PRODH/POX leads to the production of ATP for survival, in the second one, ROS induce apoptosis. Although the mechanism for the switch between apoptosis/survival is not well understood, it has been postulated that the PRODH/POX-induced apoptosis or survival is a metabolic context-dependent process and proline availability for PRODH/POX-dependent functions may play a key role.

In this report it has been hypothesized that estrogens could play an important role in the mechanism of PRODH/POX-dependent apoptosis/survival as stimulators of collagen biosynthesis, that utilize a large amount of free proline, limiting substrate (proline) availability for PRODH/POX-dependent functions. Estrogens are implicated in collagen metabolism as stimulators of collagen biosynthesis. This process is accompanied by collagen degradation finalized by cytoplasmic imidodipeptidase, prolidase.

To explore the hypothesis four breast cancer cell models were used: ER-positive MCF-7 breast cancer cell line (expressing ERα and ERβ) and ER-negative MDA-MB-231 breast cancer cell line (expressing only ERβ) and respective shRNA PRODH/POX silenced clones. To up-regulate PRODH/POX, troglitazone (TGZ), the ligand of peroxisome proliferator-activated receptor-γ (PPAR-γ), known to stimulate PRODH/POX, was used.

It has been found that estrogens stimulate collagen biosynthesis by utilizing free proline and limiting its availability for PRODH/POX-dependent apoptosis. Interestingly, TGZ was found not only as a strong PRODH/POX activator but also an inhibitor of collagen biosynthesis. It has been documented that apoptosis (activated caspase-3, -9, and PARP) was highly pronounced in wild-type MDA-MB-231 cells cultured in the medium without estradiol or in the cells cultured in the medium with estradiol but deprived of ERβ (by ICI-dependent degradation), while in PRODH/POX-silenced cells the process was not found. PRODH/POX-induced apoptosis in these cells

was reactive oxygen species (ROS) dependent. The effect was not found also in MCF-7 cells independently of the absence or presence of estradiol and in MDA-MB-231 cells cultured in the medium with estradiol. The mechanism for the process was found at the level of collagen biosynthesis, the most effective process of proline utilization, that is up-regulated by estrogens. The data suggest that TGZ-induced apoptosis in MDA-MB-231 cells cultured in the medium without estradiol or deprived of ERβ is mediated by PRODH/POX and the process is facilitated by proline availability for PRODH/POX by TGZ-dependent inhibition of collagen biosynthesis. It suggests that combined PPAR-γ agonist and antiestrogen treatment could be considered in experimental therapy of estrogen receptor-negative breast cancers.

STRESZCZENIE

Niniejsza rozprawa doktorska dostarcza dowodów ważnej roli estrogenów w mechanizmie apoptozy zależnej od PRODH/POX w komórkach raka piersi.

PRODH/POX, enzym mitochondrialny, katalizuje konwersję proliny do kwasu Δ1-pirolino-5-karboksylowego (P5C). Podczas tego procesu uwolnione elektrony są transportowane do łańcucha oddechowego, wytwarzając ATP lub są bezpośrednio przyjmowane przez tlen, generując reaktywne formy tlenu (ROS). W pierwszym przypadku aktywacja PRODH/POX prowadzi do produkcji ATP kreując warunki pro-przeżyciowe, w drugim ROS indukują apoptozę. Jakkolwiek mechanizm przełączania funkcji apoptozy/przeżycia nie jest dobrze poznany, postuluje się, że obydwa procesy (apoptoza lub przeżycie) wywołane przez PRODH/POX są zależne od kontekstu metabolicznego komórki, a dostępność proliny do PRODH/POX-zależnych funkcji może odgrywać kluczową rolę.

Wysunięto hipotezę, że estrogeny mogą odgrywać ważną rolę w mechanizmie apoptozy/przeżycia zależnej od PRODH/POX jako stymulatory biosyntezy kolagenu, który to proces utylizuje duże ilości wolnej proliny, ograniczając dostępność substratu (proliny) do PRODH/POX zależnych funkcji. Estrogeny są bowiem stymulatorami biosyntezy kolagenu. Procesowi temu towarzyszy degradacja kolagenu, która w końcowym etapie katalizowana jest przez cytoplazmatyczną imidodipeptydazę, prolidazę, uwalniając prolinę.

Do zbadania powyższej hipotezy wykorzystano cztery modele komórek raka piersi: ER-pozytywną linię komórkową raka piersi MCF-7 (z ekspresją ERα i ERβ) oraz ER-negatywną linię komórkową raka piersi MDA-MB-231 (z ekspresją tylko ERβ) a także odpowiednie linie komórkowe z wyciszoną ekspresją PRODH/POX uzyskane metodą shRNA. Aby pobudzić ekspresję PRODH/POX, zastosowano troglitazon (TGZ), ligand receptora aktywowanego przez proliferatory peroksysomów (PPAR-γ), o znanej zdolności do stymulacji ekspresji PRODH/POX.

Stwierdzono, że estradiol stymuluje biosyntezę kolagenu utylizując wolną prolinę i ograniczając jej dostępność do apoptozy zależnej od PRODH/POX. Co ciekawe, TGZ okazał się nie tylko silnym aktywatorem PRODH/POX, ale także inhibitorem biosyntezy kolagenu. Przedstawiono dowody, że apoptoza (aktywna kaspaza-3, -9 i PARP) zachodzziła w komórkach MDA-MB-231 typu dzikiego,

hodowanych w pożywce bez estradiolu lub w komórkach hodowanych w pożywce z estradiolem, ale pozbawionych ERβ (przez Degradacja zależną od ICI), podczas gdy w komórkach z wyciszonym PRODH/POX apoptozy nie stwierdzono. Apoptoza w tych komórkach wywołana przez PRODH/POX była zależna od reaktywnych form tlenu (ROS). Efektu tego nie stwierdzono również w komórkach MCF-7 niezależnie od nieobecności lub obecności estradiolu oraz w komórkach MDA-MB-231 hodowanych w pożywce z estradiolem. Mechanizm PRODH/POX-zależnej apoptozy jest uzależniony od intensywności biosyntezy kolagenu, najefektywniejszego procesu utylizacji proliny, który jest regulowany przez estrogeny.

Przedstawione wyniki badań sugerują, że apoptoza indukowana przez TGZ w komórkach MDA-MB-231 hodowanych w pożywce bez estradiolu lub pozbawionych ERβ zachodzi poprzez PRODH/POX, a proces ten jest nasilony przez wzrost dostępności proliny do PRODH/POX za pośrednictwem TGZ silnie hamującym biosyntezę kolagenu. Sugeruje to, że skojarzone działanie agonisty PPAR-γ i anty-estrogenu może stanowić przedmiot dalszych badań nad eksperymentalną terapią ER-negatywnego raka piersi.

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AUTHORSHIP/ CO-AUTHORSHIP STASTEMENTS

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30.05.2022, Bialystok

Statement

I declare that my contribution to the preparation of the publication:

1. Lewoniewska, S., Oscilowska, I., Forlino, A., Palka, J. Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells. Biology, 2021, 10, 1314. MSWiA:100 points, Impact Factor ISI: 5,079. DOI: 10.3390/biology10121314

that has been included in my doctoral dissertation consisted: topic of the article, figures preparation, literature, review and drafting the manuscript, which I define as 70% participation in the preparation of the above-mentioned publication.

2. Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone-Induced PRODH/POX-Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4,242. DOI: 10.3390/jcm10204641

that has been included in my doctoral dissertation consisted: design of the study, co-participation in preparation of PRODH/POX-silenced cell lines, biochemical assays, co-participation in statistical analyses, interpretation of the results and drafting the manuscript, which I define as 60% participation in the preparation of the above-mentioned publication.

Sylwia Lewoniewska
Signature of PhD thesis author

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prof. dr hab. Jerzy Pałka

Prof. dr hab. Jerzy Pałka (supervisor)

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which is a part of doctoral dissertation of Sylwia Lewoniewska, my contribution included co-participation in preparation and critical review of the manuscript.

I agree to use this publication by Sylwia Lewoniewska, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

KIEROWNIK
Zakładu
prof. dr hab. Jerzy Palka

.....
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UNIVERSITÀ DI PAVIA
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Prof. Antonella Forlino
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Statement

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Antonella Forlino

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which is a part of doctoral dissertation of Sylwia Lewoniewska, my contribution included co-participation in data analyses and biochemical assays.

I agree to use this publication by Sylwia Lewoniewska, in the procedure for awarding the doctoral degree in the field of medical sciences and health sciences in the discipline of medical sciences.

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1. Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone-Induced PRODH/POX-Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4,242. DOI: 10.3390/jcm10204641

which is a part of doctoral dissertation of Sylwia Lewoniewska, my contribution included co-participation in statistical analyses.

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P1. Lewoniewska, S., Oscilowska, I., Forlino, A., Palka, J. Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells. Biology. 2021, 10, 1314. MSWiA: 100 points. Impact Factor ISI: 5.079. DOI: 10.3390/biology10121314.

Author's name and surname	Nature of participation	Contribution in %
PhD student – MSc Sylwia Lewoniewska	Conception and design, figures preparation, literature, review and manuscript preparation	70%
Dr Ilona Oscilowska (Zareba)	Participation in preparation and review of the manuscript	10%
Prof. Antonella Forlino	Supervision, revising the manuscript	10%
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I hereby declare that all co-authors agreed to use these article in the doctoral dissertation by Sylwia Lewoniewska.

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P2. Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone- Induced PRODH/POX- Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4.242. DOI: 10.3390/jcm10204641.

Author's name and surname	Nature of participation	Contribution in %
PhD student – MSc Sylwia Lewoniewska	Conception and design, co-participation in preparation of PRODH/POX-silenced cell lines, biochemical assays, co-participation in statistical analyses, interpretation of the results, preparing the manuscript	60%
Dr Ilona Oscilowska (Zareba)	Preparation of PRODH/POX-silenced cell lines, biochemical assays, review of the manuscript	15%
MSc Thi Yen Ly Huynh	Co-participation in biochemical assays, literature collection	5%
Dr Izabela Prokop	Co-participation in data analyses and biochemical assays	5%
MSc Weronika Baszanowska	Co-participation in biochemical assays	5%
Dr Katarzyna Bielawska	Co-participation in statistical analyses	5%
Prof. Jerzy Palka	Supervision, revising the manuscript	5%

I hereby declare that all co-authors agreed to use these article in the doctoral dissertation by Sylwia Lewoniewska.

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W nawiązaniu do pisma z dn. 28.02.2022 r. dotyczącego doktoratu mgr Sylwii Lewoniewskiej o tytule: *„The role of estrogen receptor status in prolinę dehydrogenase/prolinę oxidase (PRODH/POX)-dependent apoptosis in breast cancer cells"* informuję, że nie ma w tym przypadku konieczności uzyskiwania zgody Komisji Bioetycznej.

Przedstawiony certyfikat autentyczności i czystości komercyjnej linii komórkowej gwarantuje dochowanie należytej staranności w przestrzeganiu przepisów pozyskania materiału biologicznego, jak też przestrzegania norm etycznych.

Z poważaniem

prof. dr hab. Otylia Kowal-Bielecka
Przewodnicząca Komisji Bioetycznej
przy Uniwersytecie Medycznym
w Białymstoku

SCIENTIFIC ACHIEVEMENTS

1. List of publications constituting the doctoral dissertation

— Lewoniewska, S., Oscilowska, I., Forlino, A., Palka, J. Understanding the Role of Estrogen Receptor Status in PRODH/POX-Dependent Apoptosis/Survival in Breast Cancer Cells. Biology, 2021, 10, 1314. MSWiA: 100 points, Impact Factor ISI: 5.079. DOI: 10.3390/biology10121314.

—Lewoniewska, S., Oscilowska, I., Huynh, T.Y.L., Prokop, I., Baszanowska, W., Bielawska, K., Palka, J. Troglitazone- Induced PRODH/POX- Dependent Apoptosis Occurs in the Absence of Estradiol or ERβ in ER-Negative Breast Cancer Cells. J. Clin. Med., 2021, 10, 4641. MSWiA: 140 points, Impact Factor ISI: 4.242. DOI: 10.3390/jcm10204641.

2. List of other scientific publications and monographs

— Lewoniewska, S., Szoka, Ł., Palka, J. Multifunctional potential of natural phytoestrogens, biochanin A and genistein. Advances in Biomedical Research. Wydawnictwo Naukowe TYGIEL. ISBN:978-83-67104-12-8, 2021, 193-202.

—Lewoniewska, S., Szoka, L., Palka, J. Phytoestrogen, biochanin A augments troglitazone-induced apoptosis in breast cancer cells. (Publication pending review in Applied Sciences).

—Oscilowska, I., Rolkowski, K., Baszanowska, W., Huynh, TYL., Lewoniewska, S., Niziol, M., Sawicka, M., Bielawska, K., Szoka, P., Miltyk, W., Palka, J. Proline Dehydrogenase/Proline Oxidase (PRODH/POX) Is Involved in the Mechanism of Metformin-Induced Apoptosis in C32 Melanoma Cell Line. Int Mol Sci, 2022, 23, 2354.

—Huynh, T.Y.L., Zareba, I., Baszanowska, W., Lewoniewska, S., Palka J. Understanding the role of key amino acids in regulation of proline dehydrogenase/proline oxidase (prodh/pox)-dependent apoptosis/autophagy as an approach to targeted cancer therapy. Mol Cell Biochem, 2020, 466, 35–44.

—Koper-Lenkiewicz, O.M., Kaminska, J., Wilinska, E., Milewska, A., Lewoniewska, S., Tomaszewska, J., Zinczuk, J., Dymicka-Piekarska, V., Matowicka-Karna, J. Factors associated with erythrocyte count and hemoglobin concentration in men and women with type 2 diabetes. Diagn Lab, 2019, 55(3), 199-208.

—Zinczuk, J., Lewoniewska, S., Zareba, K., Pryczynicz, A., Guzinska-Ustymowicz, K. Glucagonoma as a rare case of neuroendocrine tumor of the pancreas: a case report. Prog Health Sci, 2019, 9(1), 169-173.

—Koper-Lenkiewicz, O.M., Kaminska, J., Lewoniewska, S., Wilinska, E. The role of the blood-cerebrospinal fluid barrier and the blood-brain barrier in maintaining homeostasis of the central nervous system. Polski Przegląd Neurologiczny, 2018, 14(4), 200-208.

—Lewoniewska, S., Baszanowska, W., Misiura, M., Niziol, M., Zareba, R., Huynh, TYL., Zareba, I., Rysiak, E., Prokop, I. The role of preventive examinations among working people. Problemy promocji zdrowia. P-ISBN:978-83-958710-3-0, 2020, 223-232.

—Misiura, M., Baszanowska, W., Lewoniewska, S., Huynh, TYL., Niziol, M., Zareba, R., Oscilowska, I., Rysiak, E., Rolkowski, K. Application of natural products in primary prevention of cancer. Sytuacje trudne w ochronie zdrowia. P-ISBN:978-83-960390-6-4, 2020, 346-353.

—Lewoniewska, S., Zareba, R., Baszanowska, W., Misiura, M., Nizioł, M., Prokop, I., Rysiak, E., Zareba, I. Transplants as a bioethical problem. Polski przegląd nauk o zdrowiu, 2019, 60(3), 216-220.

—Baszanowska, W., Misiura, M., Ró łkowski, K., Lewoniewska, S., Huynh, T.Y., Zaręba, R., Baszanowska, H., Prokop, I., Rysiak, E., Zaręba, I. Hypoglycemic mechanisms of antidiabetic drugs action. Polski Przegląd Nauk o Zdrowiu. 2019, 3(60):210–215.

—Baszanowska, W., Lewoniewska, S., Misiura, M., Huynh, TYL., Zareba, R., Baszanowska, H., Prokop, I., Rysiak, E., Oscilowska, I., Rolkowski, K. Cosmetology in oncology. Sytuacje

trudne w ochronie zdrowia. P-ISBN:978-83-960390-6-4, 2019, 295-302.

—Lewoniewska, S., Rolkowski, K., Zareba, I. Diabetes diagnosis. Gazeta Farmaceutyczna, 2019, 28(7), 10-12.

—Lewoniewska, S., Rolkowski, K., Zareba, I. Diabetes complications part 2. Gazeta Farmaceutyczna, 2019, 28(10), 16-18.

3. List of congress reports

— Huynh, T.Y.L., Baszanowska, W., Lewoniewska, S., Ościłowska, I., Pałka, J. Proline dehydrogenase/ Proline Oxidase knockdown inhibits p53dependent apoptosis in MCF-7 breast cancer cells. EMBO Workshop "Systems approaches in cancer". (21-26.09.2021) Split, Croatia.

—Lewoniewska, S., Szoka, L., Palka, J. Natural anti-estrogens augment proline oxidase-dependent apoptosis in breast cancer cells. Flash talk at 15-th International Scientific Conference „The Vital Nature Sign" (20-21.05.2021) Kaunas, Lithuania.

—Lewoniewska, S., Oscilowska, I., Huynh, TYL., Baszanowska, W., Prokop, I., Palka, J. The impact of estrogen receptor activation on troglitazone- induced apoptosis in breast cancer cells. Poster presentation at 4th ENABLE „Exploring Life Dynamics: in and out of equilibrium" (12-14.05.2021) Milan, Italy.

—Lewoniewska, S., Prokop, I., Huynh, TYL., Baszanowska, W., Zareba, I. Estrogens regulate PRODH/POX-dependent apoptosis. Poster presentation at III Poznan Conference „Contemporary pharmaceutical and biomedical analytics in health care" (05-06.05.2020) Poznan, Poland.

—Baszanowska, W., Huynh, T.Y.L., Lewoniewska, S., Zaręba, I., Pałka, J. Metformina indukuje PRODH/POX-zależną apoptozę/autofagię w komórkach raka piersi MCF-7. III Poznańska Konferencja "Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia". (04-05.05.2020), Poznań, Poland.

—Huynh, T.Y.L., Baszanowska, W., Lewoniewska, S., Zaręba, I., Pałka, J. The

mechanism for proline dehydrogenase/proline oxidase-dependent regulation of apoptosis/autophagy in MCF-7 breast cancer cells. III Poznańska Konferencja "Współczesna analityka farmaceutyczna i biomedyczna w ochronie zdrowia". Poznań, konferencja on-line, (04-05.05.2020), 77.

—Huynh, T.Y.L., Baszanowska, W., Lewoniewska, S., Zaręba, I., Pałka, J. Down-regulation of p53 expression in proline dehydrogenase/proline oxidase (PRODH/POX) - dependent pathways in MCF-7 breast cancer cell. 2019 International Conference: Korean Society for Molecular and Cellular Biology (KSMCB), (2019.09.30-10.02), 134; Seoul, Korea.

—Lewoniewska, S., Baszanowska, W., Huynh, TYL., Zareba, I., Petelska, E., Surazynski, A., Palka, J. AMPK induces apoptosis/autophagy in breast cancer MCF-7 cells through proline dehydrogenase/proline oxidase (PRODH/POX). Poster presentation at XVIII International Congress of Medical Sciences (09-12.05.2019), Sofia, Bulgary.

—Zaręba, I., Baszanowska, W., Lewoniewska, S., Huynh, T.Y.L., Prokop, I., Rysiak, E., Pałka Jerzy. Down-regulation of estrogen receptor function facilitate proline dehydrogenase/proline oxidase-dependent apoptosis in MCF-7 breast cancer cells. XVIII International Congress of Medical Sciences. 09-12 may 2019, 63; Sofia, Bulgaria.

—Baszanowska, W., Huynh, T.Y.L., Zaręba, I., Lewoniewska, S., Cywoniuk, A., Petelska, E., Pałka, J. Metformin inhibits collagen biosynthesis facilatating proline availability for PRODH/POX-dependent apoptosis/autophagy in MCF-7 breast cancer cells. XVIII International Congress of Medical Sciences. 09-12 may 2019, 87; Sofia, Bulgaria.

—Huynh, T.Y.L., Lewoniewska, S., Baszanowska, Weronika, ., Zaręba, I., Pałka, J. POX-dependent inhibition of P53 expression in MCF-7 creast cancer cell. XVIII International Congress of Medical Sciences. 09-12 may 2019, 85; Sofia, Bulgaria.

—Lewoniewska, S., Wilinska, E., Koper, O.M., Kaminska, J. Diagnostic etility of CXCL8

index in patients with gliomas. Oral presentation at I National Neurology Conference (11.11.2017) Olsztyn, Poland- **First avard.**

—Wilinska, E., Lewoniewska, S. Evaluation of CCL2/MCP-1 levels in cerebrospinal fluid and serum of patients with gliomas. III National Conference of Laboratory Medicine (20.05.2017) Sosnowiec, Poland, **Avarded**.

4. List of other scientific activities

4.1. Research projects:

—**Co-investigator of research project financed from the National Science Center, Medical University of Bialystok, Poland (2018-2022)**
(Project No:N/NCN/OP/18/001/2214)
Project title: „Identification of the mechanism of anticancer activity of metformin as an experimental approach to improve cancer pharmacotherapy"

—**Leader of research grant, Medical University of Bialystok, Poland. (2019-2021)**
(Project (No POWR.03.02.00-00-I051/16 co-funded from European Union funds,
PO WER 2014-2020 grant 06/IMSD/G/2019).
Project title: „The role of anti-estrogens in regulation of POX-dependent apoptosis in MCF-7 breast cancer cells"

—**Leader of the statutory project, Medical University of Bialystok, Poland (2019-2020)**
(Project No: SUB/2/DN/19/001/2214)
Project title: „Evaluation of the mechanism of pro-apoptotic action of metformin in melanoma cells"

—**Co-investigator of the statutory project, Medical University of Bialystok, Poland (2019-2020)**
(Project No: SUB/2/DN/19/002/2214/2020) Project title: „ Evaluation of the mechanism of pro-apoptotic action of vitamin D3 and vitamin K in melanoma cells"

4.2.Scientific internships/ training courses:

—Training ,,Fully humanized mouse models for Immuno-Oncology preclinical drug candidate selection. Online course, California, United States (29.11.2021)

—Training ,,Project management in practice - planning, controlling, managing time, change and communication using practical and proven tools", SEMPER Training and Conference Centre, Poznan, Poland (04-05.11.2021)

—Training ,,Fluorophore fundamentals for flow cytometry", Thermo Fisher Scientific, online, California, United States, (02.11.2021)

—Training ,,Applying design principles to schematic figures", EMBO, Heidelberg, Germany (22.10.2021)

—Training ,,Communicating research: paper writing and short presentations", EMBO, Heidelberg, Germany (20-21.10.2021)

—Training ,,Selected issues in laboratory diagnostics of chronic liver diseases", Medical Centre for Postgraduate Education, Warsaw, Poland (14-15.10.2021)

—Training ,,Selected issues from molecular basis of oncogenesis", Medical Centre for Postgraduate Education, Warsaw, Poland (07.10.2021)

—Summer school ,,Molecular Biology Summer Workshop", New England Biolabs, Northampton, United States (25.07-6.08.2021)

—Training ,,Real-time PCR- applications in biology and medicine", Medical Centre for Postgraduate Education, Warsaw, Poland (16.06-17.06.2021)

—Summer school ,,Scientific discourse and modern research technologies and scientific success", Medical University of Bialystok, Poland (06.2021)

—Summer school ,,Biotechnology in medicine and pharmacy", Vytautas Magnus University, Kaunas, Lithuania (09.2020)

—Course online. Lumit™ Immunoassays: An Easier, Faster Method for Analyte Detection. Promega (13.05.2020)

—Internship in Center for Neuroscience and Cell Biology, University of Coimbra, Portugal (01.10-30.11.2019)

—Summer school ,,Soft skills and academic success", Suprasl, Poland (03-07.06.2019)

—Course ,,Cell cultures-fundamentals, applications and practical advice", Promega, Poland (29.05.2019)

—Course ,,Animal models and cell culture", AnimaLab, Bialystok, Poland (12.03.2019)

—Course ,,Application of spectrometric methods in environmental and forensic research", Ms Spectrum, Bialystok, Poland (13.11.2018)

—International course of Neuroscience for PhD students ,,Synapses, neuronal circuits and behavior" supported by Federation of European Neuroscience Societies, International Brain Research Organization, Coimbra, Portugal (09-20.04.2018)

—Internship in Center for Neuroscience and Cell Biology, University of Coimbra, Portugal (09.04-09.07.2018)

LIST OF FIGURES

fulvestrant (ICI 182 780) in the presence (7C, 7G and 7I) and absence (7D, 7H and 7J) of estradiol.

Figure 8. Western blot for cleaved caspase-3, PARP, caspase-9 and p53 in wild type and PRODH/POX-silenced (shPRODH/POX) MDA-MB-231 cells treated with troglitazone (TGZ) in the presence (A) and absence (B) of estradiol. GAPDH expression was used as a loading control. The Western blot bands intensity of representative blots were quantified by densitometry and normalized to GAPDH (Supplementary Material, SFigure 12-SFigure 15).

Figure 9. Western blot for cleaved caspase-3, PARP, caspase-9 and p53 in wild type and PRODH/POX-silenced (shPRODH/POX) MCF-7 cells treated with troglitazone (TGZ) in the presence (A) and absence (B) of estradiol. GAPDH expression was used as a loading control. The Western blot bands intensity of representative blots were quantified by densitometry and normalized to GAPDH (Supplementary Material, SFigure 8-SFigure 11).

Figure 10. Proline concentration (A, B), collagen biosynthesis (C, D) and prolidase activity (E, F) in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MCF-7 cells stimulated by troglitazone (TGZ) in the presence (A, C, E) and the absence (B, D, F) of estradiol.

Figure 11. Proline concentration (A, B), collagen biosynthesis (C, D) and prolidase activity (E, F) in wild type (wt of PRODH/POX) and PRODH/POX-silenced (sh of PRODH/POX) MDA-MB-231 cells stimulated by troglitazone (TGZ) in the presence (A, C, E) and the absence (B, D, F) of estradiol.