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Doxorubicin affects expression of the *ZFP36* and *CTTN* genes in MCF7 cell line

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Doxorubicin (DXR) is used in breast cancer therapy, and may change transcriptional profile of cells. Tristetraprolin (TTP) is an mRNA-decay protein, which is downregulated in cancer, inhibits invasion and is suggested as a positive prognostic marker. Migration and invasion require the presence of TKS4, TKS5, N-WASP, WIP and CTTN proteins. The investigation of how doxorubicin may regulate these targets is important for understanding its impact on tumor. **Aim.** The study aims to investigate the effect of doxorubicin treatment of MCF7 cells on the expression of *ZFP36, SH3PXD2A, CTTN, SH3PXD2B, WIPF1* and *WASL* genes coding for TTP, TKS4, TKS5, N-WASP, WIP and CTTN proteins correspondingly. **Methods.** Cell culture, RT-qPCR, western-blotting and Alamar Blue assay are used in this study. **Results.** DXR increases both mRNA and protein levels of TTP, which may limit its use as a prognostic marker; it also affects the expression of *CTTN*, but does not affect *SH3PXD2A*, *SH3PXD2B*, *WIPF1* and *WASL*. The observed effect is likely an overall response to DNA damage. **Conclusion.** Doxorubicin significantly affects the expression of *ZFP36* and *CTTN* genes in MCF7 cells, and further investigations are needed to reveal its effects on the cancer cells physiology.

Keywords: doxorubicin, breast cancer, TTP

Introduction

Breast cancer (BC) is one of the most common malignancies in women, and still remains a significant global health concern, with millions of cases diagnosed worldwide. In 2022, breast cancer continued to pose a significant health burden globally, with 2.3 million new cases diagnosed worldwide and accounted for an estimated 685,000 deaths globally [1].These numbers highlight the ongoing importance of raising awareness, improving screening pro-

grams, and advancing treatment options to reduce the impact of breast cancer on individuals and communities worldwide.

BC is a highly heterogeneous disease, and traditionally is divided by 4 subtypes based on progesterone, estrogen and HER2 receptor status and the presence or absence of other marker molecules. Luminal A is a hormone receptor-positive, HER2-negative, typically slow-growing; Luminal B is also hormone receptor-positive, but with higher proliferation markers or HER2 overexpression; HER2-positive is characterized by overexpression or amplification of HER2 receptor, is aggressive and requires targeted therapies; and finally is triple-negative, that lacks hormone receptors and HER2/neu overexpression, aggressive with limited treatment options [2]. Depending on a subtype, several strategies are used in BC therapy. If the hormone receptor-positive subtypes may be treated using hormonal therapy, the triple-negative breast cancer is usually treated with chemotherapy. However, even receptor-positive tumors are often additionally treated with chemotherapeutics, such as doxorubicin (DXR), since the adjuvant therapy is used not only to inhibit the tumor progression, but also to avoid metastases. In the current study we focus on the MCF7 cell line, which corresponds to the least aggressive, but the most abundant luminal A molecular subtype [3].

The metastases formation is the leading cause of cancer-associated mortality, since it leads to recurrence with subsequent distant organ disrupting and death [4]. One of the key features of metastases is the ability of cancer cells to detach from primary tumor, spread to adjacent tissue via extracellular matrix (ECM) degradation and invade lymphatic and/or blood vessels to reach distant tissues with subsequent initiation of secondary tumor formation. Such a phenotype is usually realized via specialized actin-rich protrusions called podosomes and invadopodia [5].

The podosomes and invadopodia formation requires reorganization of actin cytoskeleton, a well-orchestrated basic cellular mechanism, that involves numerous proteins. These proteins may be divided in several categories: actin nucleation promoting factors, polymerization factors, filament stabilization and destabilization factors, as well as scaffold proteins, GTPases, kinases, proteases and integrins, which assemble and activate the podosomes and invadopodia formation [6, 7]. The ability of cancer cells to overcome tight transcriptional control of pro-survival and pro-invasive genes, including cytoskeleton-associated ones, may be facilitated by the altered stability of target mRNAs. Whilst in the healthy cells the optimal level of mRNA expression of genes associated with the survival signaling cascades is maintained through strict transcriptional and post-transcriptional mechanisms, in the malignant cells, the mRNAs of many categories of pro-survival genes, including proto-oncogenes, tumor suppressors, and cytokines, are abnormally stable, leading to disruption of the normal cell cycle and affecting overall cell fate. A large number of such mRNAs contain AU-rich elements in their 3'-UTRs and can be regulated by specific RNA-binding proteins, such as tristetraprolin [8].

Tristetraprolin (TTP, encoded by *ZFP36* gene) is an RNA-binding protein, that belongs to the tristetraprolin family. These proteins play a key role in the post-transcriptional regulation of a target gene expression by specifically binding to the AU-rich elements of their mRNAs.

They are considered to be the key regulators of many cellular processes associated with malignancy, and their expression is dysregulated in many types of cancer, including human cancers. They often have suppressive effects on tumors and are also associated with the patient survival prognosis, metastasis, and resistance to chemotherapy [9]. However, it is not known whether the expression of TTP may be impaired during treatment and how it may be involved in the tumor cells' fate.

Doxorubicin belongs to one of the most widely used chemotherapeutics in BC, and different therapeutic strategies may include doxorubicin separately or in combination with other hormonal and/or antineoplastic agents [2]. DXR is an anthracycline antibiotic known to intercalate DNA, causing genotoxic stress, that eventually leads to the death of rapidly dividing cells [10]. It is also known to be a highly cardiotoxic compound, which leads to limitations of its dosage during chemotherapy [11]. Multiple studies are conducted in order to reveal the molecular mechanisms of its cardiotoxicity, showing that it may impair the expression profile of cardiomyocytes.

Since DXR is a genotoxic agent, that impairs multiple signaling pathways, we hypothesize that it may also lead to altered expression of TTP, as well as of the genes involved in the cytoskeleton organization. We attempt to reveal whether such an effect occurs in the MCF7 breast cancer cell line.

Materials and Methods

Cell culture and treatment

MCF7 cells were grown in Dulbecco's modified Eagle medium with 4500 mg/L glucose (Gibco, #11965092), 10 % fetal bovine serum (FBS), and penicillin/streptomycin at 37 °C with 5 % CO_2 in a humidified incubator. When they reached approximately 70–80 % confluence, they were exposed to 0.1, 0.5, or 1.0 μM of DXR (Sigma, D1515), and then cultured for 48 hours. Subsequently, they were trypsinized, washed twice with cold PBS, and divided into two microcentrifuge tubes: one for RNA isolation and the other for protein lysate preparation. All the samples were handled on ice. All the experiments were performed as two independent experiments, repeated in technical triplicates.

Western-blotting

Protein lysates were prepared in RIPA buffer containing EDTA-free Halt protease and phosphatase inhibitors cocktail (Thermo Scientific, #78425). Protein concentrations were determined using the BCA assay (Pierce, #23225), and equal amounts of protein (e.g., 10 μg) were loaded onto NuPAGE Bis-Tris 4–12 % gels (Invitrogen, NP0322BOX). Following electrophoresis, the samples were transferred to nitrocellulose membranes using the Trans-Blot Turbo system (BioRad, #1704270). The membranes were then blocked with 5 % BSA for 1 hour, incubated overnight with primary antibody (anti-TTP, Cell Signaling, #71632, 1:1000), and subsequently incubated with secondary antibody (anti-rabbit, Jackson ImmunoResearch, AB_10015282, 1:15000). Signal detection was achieved using ECL detection reagent (Amersham, RPN2105) and documented with a ChemiDoc device (BioRad). Following imaging, the membrane was washed three times with TBST and treated with 30 % hydrogen peroxide to inhibit peroxidase activity, as described by Sennepin *et al.* [12]. Subsequently, the membrane was washed again

with TBST and incubated with primary antibody against b-tubulin (Sigma, T4026, 1:10000), followed by incubation with secondary antibody (anti-mouse Jackson ImmunoResearch, AB_10015289, 1:20000), and further signal development and documentation.

Quantitative RT-PCR

RT-qPCR was performed using LUNA Universal One-Step RT-qPCR master mix (NEB, E3005). Equal amount of RNA, isolated using RNAeasy mini Plus kit (Qiagene, #74134), was loaded to each reaction. Efficiency of primers was calculated for each primer pair (see primer sequences and efficiencies in Table 1). Relative gene expression was calculated using the following equation, with *GAPDH* as a reference gene:

$$RelExp = (E_{GOI})^{\Delta Ct_{(GOI)}} / (E_{HKG})^{\Delta Ct_{(HKG)}}$$

where RelExp — relative expression, E_{GOI} — primers efficiency for the gene of interest, $\Delta Ct_{(GOI)}$ — ΔCt for the gene of interest, E_{HKG} — primers efficiency for the reference gene, $\Delta Ct_{(HKG)}$ — ΔCt for the reference gene.

Alamar Blue cytotoxicity assay

To assess the cytotoxicity of DXR, the cells were plated in 96-well plates at a density of 10,000 cells per well and allowed adhering overnight. Subsequently, the cells were treated with 0.1, 0.5 or 1.0 μM of DXR and incubated for 72 hours. Then, the cells were washed with PBS, and fresh medium containing 10 % alamarBlue reagent was added. Following a 2-hour incubation period, the absorbance was measured using a microplate reader. Untreated cells served as the control group.

Statistical analysis

For statistical analysis we used PrismGraphPad 9.5 software. ANOVA was used for multiple group comparison, and two-tailed t-test for pairwise comparison. Correlation was calcu-

Table 1. **Sequence and efficiency of the used primers**

Primer pair	Sequence (5'-3')	Efficiency
For-ZFP36 (TTP) Rev-ZFP36 (TTP)	TCTTCGAGGCGGTTTTT TGCGATTGAAGATGGGGAGTC	93.5 %
For-SH3PXD2A Rev-SH3PXD2A	CGAACCTACGGACAAGACCTC CGTGGCTTTGGCAGTTGGAA	93.78 %
For-SH3PXD2B Rev-SH3PXD2B	GGCTGTCAAACGCCTGATAC GGTTTGGTCACCCCCAGATTT	100.82 %
For-WIPF1 Rev-WIPF1	ACGGCCAACAGGGATAATGAT GGTTTCGCAGATGTGGATCTT	102.13 %
For-WASL Rev-WASL	GAACGAGTCCCTCTTCACTTT TTCCGATCTGCTGCATATAACT	105.8 %
For-CTTN Rev-CTTN	GCTTTGAGTATCAAGGCAAAACG CCAACGGCACATTTGTCTTGT	90.13 %
For-GAPDH Rev- GAPDH	AGCCACATCGCTCAGACAC GCCCAATACGACCAAATCC	101.3 %

lated using Spearman correlation coefficient. Confidence level was set at 0.05. All the graphs depict mean ± standard deviation. * represents $p < 0.05$, ** represents $p < 0.01$.

Results and Discussion

Previously, Lee and colleagues showed that the treatment with resveratrol, a natural compound with anti-proliferative effect, induces expression of TTP in colorectal cancer cells and inhibits their proliferation and metastasis [13]. Additionally, there are 2 studies, investigating the effects of DXR on TTP expression in different cell lines, including MCF7 [14, 15]. The authors investigated the effects of high concentrations of doxorubicin (from 1.5 to 3 μM) after 24 hours of incubation and found that it increased TTP expression. However, since clinically relevant concentrations of doxorubicin are suggested to be up to 1 μM, we intended to check if DXR, as a genotoxic agent, might influence the expression of TTP in lower concentration but with longer exposure (48h). Moreover, DXR might also affect expression levels of the genes, involved in the cytoskeleton reorganization and invasion. There are the studies showing that DXR impairs actin cytoskeleton function, mostly via stimulating oxidative stress and affecting signaling pathways, such as ROCK1 or RHOA [16, 17]. However, up to date there are no coherent data about the DXR effects on the expression levels of genes, included in our study. The analysis of publicly available transcriptomic databases showed no data on the expression of target genes under similar experimental conditions.

To check our hypothesis, we performed quantitative qPCR on the MCF7 cells, treated with clinically relevant concentrations of DXR, to measure expression levels of *ZFP36*, as well as expression of *SH3PXD2A, SH3PXD2B, CTTN, WIPF1* and *WASL* genes, which are associated with cell motility and invasion.

Analysis showed that lower concentrations of DXR, 0.1 and 0.5 μM, did not significantly change *ZFP36* expression, but treatment with 1.0 μM DXR resulted in a nearly 2-fold increase in mRNA level (Fig. 1, A). However, Western blot analysis showed the highest level of TTP protein at 0.5 μM concentration of DXR (Fig.1, B). We hypothesize it might be caused by the known TTP's ability to negatively regulate its own mRNA, which may lead to a poor correlation between the mRNA and protein product levels [18].

Tristetraprolin is often considered a good prognostic marker in various cancers, such as breast, prostate, hepatic, and other human cancers: it is shown that high TTP expression levels are associated with extended survival rates and less aggressive tumor behavior [19]. However, since our results indicate a DXR-dependent induction of TTP expression, we suggest that in a cohort of patients with luminal A breast cancer treated with DXR, it would be challenging to determine whether its high expression reflects a good physiological condition or is caused by the therapy. Used here concentrations of DXR correspond to peak plasma concentrations, observed in patients with breast cancer [20, 21]. Thus, our data may be with caution extrapolated to *in vivo* conditions, indicating that at least in the cohort of patients with luminal A breast cancer it will be difficult to distinguish DXR-induced increase of *ZFP36* expression from its high physiological level, thereby limiting its utility as a prognostic marker. Undoubtedly, this sugges-

tion warrants further investigation into the ZFP36 levels in patients treated with DXR compared to a control group.

Complex regulation of all cellular processes requires numerous adapter/scaffold proteins, that provide protein-protein interactions, forming protein complexes for the wide range of the crucial processes. Among others, such proteins include members of the TKS family TKS4 and TKS5, encoded by *SH3PXD2A* and *SH3PXD2B* genes, respectively, and verprolin WIP, CTTN, and N-WASP, encoded by *WIPF1*, *CTTN* and *WASL* genes. TKS proteins act as a platform for recruiting essential signaling molecules. Their structural characteristics enable the binding and anchoring of these proteins to the cytoplasmic membrane during EGFR signaling. Consequently, TKS proteins play a crucial role in cell motility, cancer cell invasion, ROS-dependent processes, and embryonic development [22]. Cortactin (CTTN) is an actin-binding and nucleation-stimulating factor abundant in the cell cortex and other peripheral structures of most cell types. It is involved in numerous cellular functions that require cytoskeleton remodeling, including lamellipodia formation, cell migration, invasion, and other cell type-dependent processes [23]. WIP and N-WASP are scaffold proteins that play an important role in the remodeling of the actin cytoskeleton. WIP regulates actin polymerization by linking the actin machinery to signaling cascades. WIP binds to WASP and its homolog N-WASP, which are central activators of the actin-nucleotide complex Arp2/3, regulates their cellular distribution, function and stability [24]. 80 % of WIP in the cell is complexed with N-WASP, protecting it from degradation. By binding directly to actin, WIP promotes the formation and stabilization of actin filaments [25–27].

We hypothesized that the overexpression of TTP induced by DXR, or DXR itself, might influence the expression levels of target genes. To check this hypothesis, first we assessed cytotoxicity of doxorubicin for MCF7 cells using Alamar blue assay (Fig.1, C). IC_{50} values for doxorubicin in MCF7 cells significantly vary in different studies [28–31]. The values fluctuate between 50 nM and 27 μM and may depend not only on experimental conditions, but also on different sensitivities of cells to doxorubicin across various labs, therefore the assessment of IC_{50} in each case is needed to understand the effects of cytotoxic agent on the cellular metabolism. This understanding can then inform how the observed effects might be extrapolated to particular cells. Further, we measured expression levels of these genes' in the MCF7 cells, treated with DXR, as described above. Interestingly, the analysis showed that DXR significantly induced *SH3PXD2A* level under 0.5 and 1.0 μM treatment. The only statistically meaningful increase of *CCTN* expression was observed at a DXR concentration of 0.1 μM. WIPF1 expression significantly increased at 0.5 μM compared to 0.1 μM DRX but not to untreated cells. Other targets did not exhibit significant changes in expression across concentrations. Interestingly, there was a high degree of variability in the expression levels of all targets, even when treated with the same concentration of DXR. We suggest it might be caused by high heterogeneity of MCF7 cells both in morphological and physiological features, which may contribute to different transcriptome landscape during passaging and treatment [3].

Moreover, the MCF7 cell line is characterized by the presence of p53, a protein that is activated in response to DNA damage. This activation affects multiple cellular pathways. Given that cytoskeleton reorganization is a fundamental cellular function, the impact and significance of any treatment depend on the dynamic interplay between these activated pathways. Finally, our correlation analysis of the expression levels of target genes revealed a mild, yet statistically significant, positive correlation among most of the genes (Fig. 1, D).

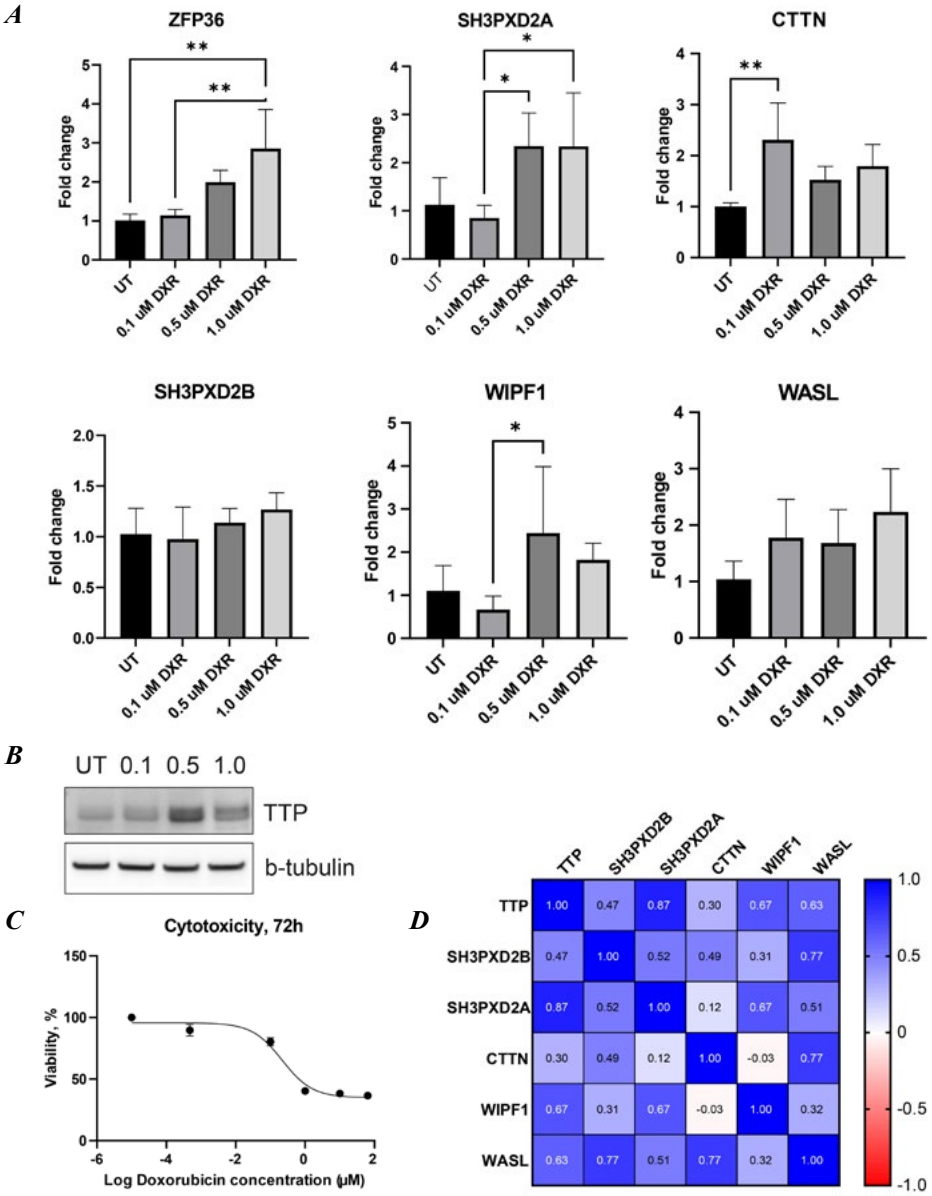

Fig. 1. Effect of DXR on expression of target genes and viability of MCF7 cells. *A* — Expression levels of ZFP36, SH3PXD2A, CTTN, SH3PXD2B, WIPF1 and WASL; *B* — Western-blot analysis of TTP protein levels under DXR treatment, UT — untreated cells, 0.1, 0.5 and 1.0 — DXR concentration, μM; *C* — Alamar Blue assay was performed to assess the cytotoxicity of DXR for MCF7 cells, IC_{50} = 0.22 μM; *D*) Correlation between target genes' expression levels under DXR treatment. Graphs represent mean ± standard deviation; * — represents $p < 0.05$, ** — represents $p < 0.01$.

These results indicate that an increase in the target genes' expression possibly reflects the general cell response to DNA damage.

Conclusion

Doxorubicin is a genotoxic agent, which affects the cell's transcriptional profile. Our data show that DXR significantly increases the expression of TTP at both protein and mRNA levels in the luminal A breast cancer model. This may limit the use of TTP as a prognostic marker, at least within the cohort of patients with luminal A breast cancer, who are administered DXR. Moreover, the study shows that DXR may increase the expression levels of invasion-associated genes, but this may be non-specific and depict the overall response of the cell to DNA damage.

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Доксорубіцин впливає на експресію *ZFP36* та *CTTN* в клітинах лінії MCF7

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Доксорубіцин (DXR) часто застосовується в терапії раку молочної залози та може змінити транскрипційний профіль клітин. Тристетрапролін (TTP, ZFP36) — РНК-зв'язувальний протеїн, експресія якого знижується в пухлинах. Відомо, що він пригнічує інвазію та вважається позитивним прогностичним маркером. Для міграції та інвазії потрібна присутність білків TKS4, TKS5, N-WASP, WIP і CTTN. Дослідження того, як DXR може регулювати ці мішені, є важливим для розуміння його впливу на пухлини. **Мета.** Вивчити вплив доксорубіцину на експресію *ZFP36, SH3PXD2A, CTTN, SH3PXD2B, WIPF1* і *WASL* у клітинах MCF7. **Методи.** Культивування клітин ссавців, кількісна ПЛР із зворотною транскрипцією, вестерн-блот аналіз і Alamar Blue-тест. **Результати.** DXR підвищує рівень експресію TTP на рівні як мРНК, так і протеїну, що може обмежити його використання як прогностичного маркера; впливає на експресію *CTTN*, але не *SH3PXD2A*, *SH3PXD2B*, *WIPF1* та *WASL1*. Такий ефект, ймовірно, є реакцією на пошкодження ДНК. **Висновки.** Необхідні подальші дослідження для вивчення впливу DXR на ракові клітини.

Ключові слова: Доксорубіцин, рак молочної залози, TTP

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