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ARTEMISININ MODULATING EFFECT ON HUMAN BREAST CANCER CELL LINES WITH DIFFERENT SENSITIVITY TO CYTOSTATICS

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Aim: **To explore effects of Artemisinin on a series of breast cancer cells with different sensitivity to typical cytotoxic drugs (doxorubicin — Dox; cisplatin — DDP) and to investigate possible artemisinin-induced modification of the mechanisms of drug resistance.** ***Materials and Methods:*** **The study was performed on wild-type breast cancer MCF-7 cell line (MCF-7/S) and its two sublines MCF-7/Dox and MCF-7/DDP resistant to Dox and DDP, respectively. The cells were treated with artemisinin and iron-containing magnetic fluid. The latter was added to modulate iron levels in the cells and explore its role in artemisinin-induced effects. The MTT assay was used to monitor cell viability, whereas changes of expression of selected proteins participating in regulation of cellular iron homeostasis were estimated using immunocytochemical methods. Finally, relative expression levels of miRNA-200b, -320a, and -34a were examined by using qRT-PCR.** ***Results:*** **Artemisinin affects mechanisms of the resistance of breast cancer cells towards both Dox and DDP at sub-toxic doses. The former drug induces changes of expression of iron-regulating proteins via different mechanisms, including epigenetic regulation. Particularly, the disturbances in ferritin heavy chain 1, lactoferrin, hepcidin (decrease) and ferroportin (increase) expression ($p \leqslant 0.05$) were established. The most enhanced increase of miRNA expression under artemisinin influence were found for miRNA-200b in MCF-7/DDP cells (7.1 ± 0.98 fold change), miRNA-320a in MCF-7/Dox cells (2.9 ± 0.45 fold change) and miRNA-34a (1.7 ± 0.15 fold change) in MCF-7/S cells. It was observed that the sensitivity to artemisinin can be influenced by changing iron levels in cells.** ***Conclusions:*** **Artemisinin can modify iron metabolism of breast cancer cells by its cytotoxic effect, but also by inducing changes in expression of iron-regulating proteins and microRNAs (miRNAs), involved in their regulation. This modification affects the mechanisms that are implicated in drug-resistance, that makes artemisinin a perspective modulator of cell sensitivity towards chemotherapeutic agents in cancer treatment.**

Key Words: **drug resistance, iron metabolism, artemisinin.**

Resistance to anticancer drugs is one of the major complications during the treatment of cancer patients because it causes low efficiency even of highly toxic treatment regimens.

There are two generally accepted forms of reduced sensitivity of cancer cells to cytotoxic drugs and hormones including initial resistance, when tumor cells are initially not sensitive to therapy because of changes in metabolism caused by mutations in key gene effectors and secondary resistance that develops because of the survival of tumor cell clones during the initial treatment [1].

Mechanisms, which are inherent to malignant cell transformation, constitute also the basis of resistance towards anticancer drugs namely: 1) violation of signaling pathways and metabolism; 2) violation of the cell cycle, reparative processes; 3) epigenetic changes; 4) mutations in the genes that encode the targets of anticancer drugs [1].

Therefore, resistance to chemotherapy and molecular targeted therapy is a serious problem that affects not only survival but also a quality of life of patients. For example, it is known that adjuvant and postoperative chemotherapy, which are used in metastatic cases of breast cancer (BC), often are ineffective. This necessitates the search for drugs that will be effective against the tumors of this localization resistant to anthracyclines and taxanes. In such cases in clinical practice combination therapy is used. However, the range of suitable drugs is narrow due to the development of multiple resistance. A promising approach in this situation is not only the search for new, less toxic and more potent chemotherapeutic agents but also the exploration of the possibility of overcoming the resistance of tumor cells to already known cytotoxic drugs. In particular, the use of low-toxic drugs that have long been used as therapeutic agents for the treatment of non-cancer pathologies and affect the altered metabolism of tumor cells is of great interest. One of these drugs is artemisinin [2].

The plant *Artemisia annua* was used in Chinese medicine before our era. In 1971, Chinese scientists isolated the active ingredient, a sesquiterpene lactone, called artemisinin, which is responsible for the anti-parasitic effect of this plant. In recent years, the drug has become the gold standard for malaria treatment [3]. In 2015 chemist Tu Youyou was awarded the Nobel Prize for discovery of artemisinin [4].

During primary testing of a new substance in experiments *in vitro*, its antitumor activity for the different cancer types, from hepatocellular carcinoma to the breast tumors was detected [5].

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Abbreviations used: BC – breast cancer; DDP – cisplatin; Dox – doxorubicin; EPR – electron paramagnetic resonance; FTH1 – ferritin heavy chain 1; HEP – hepcidin; LF – lactoferrin; SLC – ferroportin A.

In recent years, interest in artemisinin and its derivatives as antitumor drugs has increased significantly. There are a lot of reports of their successful use in clinical practice as separate medication and in combination with cytotoxic drugs [6].

Along with above mentioned, there is an evidence about the particular artemisinin impact on BC cells by one of its mechanisms of action mediated by estrogen receptor signaling pathway [7]. However, despite active research, all mechanisms of artemisinin antitumor action are not established yet. In addition, there are a lot of hypotheses about its ability to adjust the cancer cell sensitivity to cytotoxic drugs that require a more detailed study.

MATERIALS AND METHODS

Cell lines and drug treatment. The studies were conducted in an *in vitro* system on 5 cell lines of human mammary: MCF-7 — invasive breast ductal carcinoma sensitive and resistant to cytotoxic drugs; MDA-MB-231 and MDA-MB-468 — metastatic breast adenocarcinoma.

Three sub-lines of breast carcinoma MCF-7 cells, namely MCF-7/S, sensitive to cytostatics; MCF-7/Dox — resistant to doxorubicin (Dox); and MCF-7/DDP — resistant to cisplatin (DDP) were grown in Dulbecco Modified Eagles Medium (DMEM, Sigma), supplemented with recombinant human insulin (0.01 mg/ml) and 10% fetal bovine serum. MDA-MB-231 and MDA-MB-468 cells were cultured in Leibovitz's L-15 medium (Sigma) supplemented by 10% fetal bovine serum. All cultures were grown in humidified atmosphere with 5% CO_2 at 37 °C. Cells were provided by the Bank of Cell Lines of Human and Animal Tissues at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the NAS of Ukraine and described earlier [8].

Measurement of cell viability, using the MTT assay. For this purpose, cells were cultivated in 96-well plates in DMEM, supplemented with insulin (see above). The artemisinin (Sigma-Aldrich, USA), Dox (Ebewe, Austria), and DDP (Ebewe, Austria) were added to media at different concentrations and the cells were cultivated for either 24 or 48 h. For this purpose artemisinin was diluted in 0.01% DMSO in media. After this time, 10 µl of the MTT dye solution (Sigma, USA) (5 mg/ml in phosphate buffer saline) was added to the cells; the cells were incubated at the same conditions for 3 h. After centrifugation (1500 rpm, 5 min) the supernatant was removed. 100 µl of dimethyl sulfoxide (Serva, Germany) was added to each well, to dissolve formazan. The absorption was measured, using a multi-well spectrophotometer (STAT FAX 2100, USA) at a wavelength of 540 nm [9].

Total RNA isolation. Total RNA extraction was performed, using Ribozol RNA Isolation Kit (Amplisens, Russia). Concentration of RNA was measured, using NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). The purity of isolated RNA was controlled, analyzing the ratio of OD at 260/280 nm. RNA was dissolved in TE buffer and stored at −20 °C.

Single-stranded cDNA was synthesized from 100 ng of total RNA, using TaqMan® MicroRNA Kit for reverse transcription.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Preparation of reverse transcription reaction mix was performed according to manufacturer's protocol. Reverse transcription was performed at a "Tertsik" (DNA Tehnologiya, Russian Federation) thermal cycler. qRT-PCR was performed on Applied Biosystems 7900HT Fast Real-Time PCR System using TaqMan® MicroRNA primers and manufacturer's protocol.

Small nucleolar RNA RNU48 was used as an endogenous control for normalization of miRNA expression. Relative expression of the studied miRNAs was identified by comparative Ct method. Experiments were performed in triplicates for each line, and PCR was performed three times for each sample. Expression differences between the studied miRNA levels relative to control was calculated by the formula:

$$\text{Fold change} = 2^{-\Delta\Delta Ct},$$

where ΔCt (target — control) is equal to the difference between threshold cycles for miRNA (target) and the threshold cycle for RNU48 (control) (ΔCt (target — control) = Ct target − Ct control). ΔΔCt = ΔCt (experiment) − ΔCt (control).

Immunocytochemical study. The expression level of transferrin receptor (CD71), ferritin heavy chains 1 (FTH1), ferroportin A (SLC), hepcidin (HEP) and lactoferrin (LF) were estimated using the immunocytochemical method. Cells for immunocytochemical studies were grown on glass cover slips, fixed in cooled mixture of methanol:acetone (1:1) at −20 °C for 120 min, washed in phosphate buffered saline (PBS) and incubated with 1% bovine serum albumin for 20 min. Primary monoclonal antibodies: CD71 (Bioworld Technology, Minneapolis, MN, USA), FTH1 (GeneTex, Irvine, CA, USA), SLC (Abcam, USA), LF (ThermoScientific, USA) та HEP (Abcam, USA) were diluted in the blocking buffer and kept at a room temperature for one hour, followed by incubation with UltraVision LP Detection System (Lab Vision, Thermo Scientific) for 10 and 15 min; after the washing, the immune reaction was visualized by using DAB Quanto (Thermo Scientific). When immunocytochemical reactions were completed, the cells were stained with hematoxylin by Mayer for 10–15 s and placed in Faramount Aqueous Mounting Medium (DakoCytomation, Denmark). Evaluation of the results was carried out in 3 visual fields by light microscopy (× 1000, oil immersion) using the classical H-Score method:

$$S = 1 \cdot N1^{+} + 2 \cdot N2^{+} + 3 \cdot N3^{+},$$

where S is «H-Score» index; $N1^+$, $N2^+$ and $N3^+$ are numbers of cells with low, medium or high levels of marker expression.

It should be noted that in previous studies we have identified biomolecular markers that characterize the

metastatic potential and invasive activity of BC cells of abovementioned lines [10].

Low-temperature Fe(III) electron paramagnetic resonance (EPR). After 24 h of culturing, the cells were scrapped, washed in PBS, centrifuged at 1000 g for 10 min at 4 °C and the pellet was re-suspended in PBS. The suspension containing $2 \cdot 10^6$ cells was transferred into EPR tubes and immediately frozen in liquid nitrogen. The level of free iron was determined by a low-temperature EPR method [11]. Briefly, samples were maintained at –196 °C during recording of the spectra using a finger Dewar filled with liquid nitrogen. The following parameters were used for the low-temperature EPR: sweep width 1525 G; frequency 9.15 GHz; microwave power 40 mW; modulation amplitude 10.0 G and modulation frequency 100 kHz. The g-value was calculated using the standard formula:

$$g = h\nu/\beta H,$$

where h — Planck's constant; ν — frequency; β — Bohr magneton; H — external magnetic field at resonance.

Statistical analysis. Experimental data were analyzed using the Student's *t*-test. *p*-values less than 0.05 were considered statistically significant. Statistical analysis of the obtained data was performed, using the Statistica 6.0 software.

RESULTS AND DISCUSSION

We conducted determination of cytotoxic activity of the drug artemisinin in BC cell lines with the different sensitivity to DDP and Dox. We found that MCF-7/Dox cell line is the most sensitive to the artemisinin treatment, whereas MCF-7/DDP cell line the least sensitive (Table 1). Furthermore, we established that the cultivation of artemisinin at the IC30 dose caused changes in the phenotype of cells of each studied BC line. For example, MCF-7/DDP cells were characterized by shape loss and appearance of the large number of vacuoles visible under 40× magnification using the microscope. This indicates cytotoxic effect of artemisinin on these cells.

Table 1. Artemisinin cytotoxicity in B cell lines with different sensitivity to cytostatic drugs

BC cell lines	Free iron content, spins/ml	24 h		48 h	
		IC30, μg/ml	IC50, μg/ml	IC30, μg/ml	IC50, μg/ml
MCF-7/S	$0.56 \pm 0.1 \cdot 10^{16}$	2.5 ± 0.3	5.5 ± 0.6	1.1 ± 0.2	2.5 ± 0.5
MCF-7/Dox	$0.346 \pm 0.1 \cdot 10^{16}$	1.3 ± 0.7	3.7 ± 0.4	0.6 ± 0.01	1.46 ± 0.3
MCF-7/DDP	$1.15 \pm 0.1 \cdot 10^{16}$	1.9 ± 0.2	6.4 ± 0.7	1.3 ± 0.4	2.75 ± 0.5

According to the literature, one of the characteristics of tumor cells is elevated levels of iron, as it is crucial in the synthesis of DNA, proteins that transport oxygen during cell division [11, 12]. For example, by using the EPR-based assay, described in the experimental section of this paper, we determined that amount of EPR-active Fe(III) ions in different MCF-7 cell lines is between 0.346 and $1.15 \cdot 10^6$ spins/cell. The level of iron ions was found to be dependent upon the resistance degree of the cells towards chemotherapeutic agents. In particular, Dox-resistant cell line MCF-7/Dox contains the lowest Fe(III) amount, DDP-resistant cell line MCF-7/DDP contains 3.3 fold and wild type cell line MCF-7 — 1.6 fold higher amounts of Fe(III). Therefore, these three cell lines are a convenient model to validate whether the Fe(III) level correlates with cytotoxicity of artemisinin (Table 1). Previously, it has been reported by Meshnick et al. [12] that artemisinin and its derivatives have antitumor effects due to active lactone compound sesquiterpene peroxide, which interacts with iron ions and causes oxidative stress through the formation of free radicals. However, we found that the cytotoxicity was highest for the cells (MCF-7/Dox) with the lowest Fe(III) amounts, whereas that was lower for both other cell lines with higher Fe(III) amounts (Table 1). This result does not contradict the literature data due to the following considerations. The total amount of iron ions (Fe(III) + Fe(II)) in cells is tightly controlled. Therefore, the high level of Fe(III) ions indicates the low amount of Fe(II) ones. Since the latter ions are required for reductive activation of peroxo-bridge of artemisinin, it is logical that this prodrug is mostly efficiently activated in MCF-7/Dox cells with the lowest level of Fe(III) and, correspondingly, the highest level of Fe(II) ions.

Apart from its direct effect of intracellular oxidative stress, it was found that the mode of cytotoxic action of artemisinin involves many protein targets. In particular, artemisinin activates p38 MAPK-kinase cascade regardless of the oxidative stress due to inhibition of VEGF expression and cell migration. At the same time, it launches a number of anticancer pathways, including signaling via Pi3k-akt, Toll-like receptors and mediated by TGF-β. Moreover, this drug inhibits migration and invasive activity of tumor cells by reducing the level of disintegrins, metalloprotease 17 (ADAM family) and EGFR and targets TCR, CD28, CD4, CD8A and CTLA4 proteins [13]. Artemisinin and its derivatives promote activation of apoptosis and affect the level of c-MYC in cancer cells. Particularly active iron-activated dihydroartemisinin increases phosphorylation of this protein and accelerates its degradation in proteasomes [14]. This effect may be the cause of the low sensitivity of MCF-7/DDP, despite the high concentration of iron ions. In our previous study we established that this cell line is characterized by reduced expression of proapoptotic proteins, in particular, BCL-2 and Bax, involved in the development of resistance to DDP and high tolerance to DNA damage. This is explained by the fact that one of the mechanisms of DDP action is the formation of multiple single and double-stranded DNA breaks [15].

According to the literature, artemisinin has the selective cytotoxic effect on tumor cells due to changes in iron-regulating proteins expression. It is known that tumor cells, due to the high concentrations of transferrin receptors (CD71) on the plasma membrane actively absorb iron. Artemisinin covalently binds to CD71 receptors and thus selectively enters the tumor cells regardless of the tumor type [4].

The mechanism of artemisinin binding to CD71 receptors on the cell surface is similar to the mechanism

of its binding to the heme in cells of the *Plasmodium malariae*. Transferrin receptor binds transferrin-iron-ions complexes and by endocytosis enters the cell, acting as artemisinin transporter. After the degradation of endocytic vacuole, artemisinin is released and reacts with iron ions in the cytoplasm of tumor cells. One of the most significant effects of this process is critical DNA damage, which provokes apoptosis. In addition, artemisinin and its derivatives make cytoplasmic free iron unavailable for inclusion in the biological processes due to the palmityl accession from the lipid membrane of endocytic vacuoles [5].

Based on the facts mentioned above, one of the mechanisms of the artemisinin impact on tumor cells is modified metabolism of iron proteins. Therefore, we have investigated changes in expression of proteins involved in iron metabolism, response to redox stress and the formation of resistance to cytotoxic drugs using the immunocytochemical method. We found out that artemisinin treatment of MCF-7 cells, sensitive and resistant to Dox and DDP, causes a decrease in expression of proteins such as LF, FTH1, and HEP. Also, we established the increase of SLC expression. We did not observe any significant changes in the key effector of artemisinin response — CD71. Established increase in the levels of expression of iron metabolism proteins in MCF7/DDP cells compared to other lines are considered to be one of the possible mechanisms of tolerance to high artemisinin concentrations of this line (Table 2).

Table 2. Changes in the molecular profile of BC cells caused by cultivation with artemisinin

BC cell lines	CD71	FTH1	LF	HEP	SLC
MCF-7/S	72.0 ± 3.1	195.0 ± 3.7	251.0 ± 2.2	136.0 ± 2.3	83.0 ± 3.4
MCF-7/S+A	70.0 ± 2.7	150.0 ± 2.7*	213.0 ± 2.3*	94.0 ± 3.4*	130.0 ± 3.1*
MCF-7/Dox	84.0 ± 2.5	79.0 ± 2.1	67.0 ± 1.9	207.0 ± 2.7	200.0 ± 4.2
MCF-7/Dox+A	80.0 ± 5.8	49.0 ± 1.5*	62.0 ± 2.5*	189.0 ± 4.3*	214.0 ± 2.7*
MCF-7/DDP	215.0 ± 3.8	296.0 ± 3.3	105.0 ± 3.7	220.0 ± 2.3	203.0 ± 1.7
MCF-7/DDP+A	220.0 ± 3.2	236.0 ± 2.5*	84.0 ± 2.4*	156.0 ± 1.3*	212.0 ± 3.1*

Note: A – artemisinin; $^*p \leqslant 0.05$ compared with cell lines without artemisinin.

Since substantial changes in the expression of iron metabolism proteins were observed upon the cell treatment with artemisinin, we conducted the cultivation of MCF-7 cell cultures with a mixture of artemisinin and iron-containing magnetic fluid. The latter reagent is a source of iron ions and was used to increase amount of iron ions in cells. In this experiment we tested the hypotheses of iron-dependent artemisinin activation in cancer cells [15–17]. In particular, we discovered that the magnetic fluid in a concentration of 30 mg/ml greatly increases the sensitivity of BC cells to artemisinin (Tables 1 and 3). Thus, in combination with the low toxicity agent (iron-containing magnetic fluid) artemisinin can be considered as a potent anticancer agent.

Table 3. Artemisinin cytotoxicity in BC cell lines with different sensitivity to cytostatic drugs after treatment with iron-containing magnetic fluid in the dose of 30 μg/ml

BC cell lines	24 h		48 h	
	IC30, μg/ml	IC50, (μg/ml)	IC30, μg/ml	IC50, μg/ml
MCF-7/S	1.31 ± 0.11*	1.12 ± 0.29*	0.75 ± 0.22*	1.3 ± 0.1*
MCF-7/Dox	0.52 ± 0.23*	0.60 ± 0.16*	0.42 ± 0.01*	0.77 ± 0.05*
MCF-7/DDP	0.60 ± 0.15*	1.36 ± 0.35*	0.98 ± 0.31*	1.35 ± 0.24*

Note: $^*p \leqslant 0.05$ compared with cell lines without magnetic fluid treatment.

It is known that BC cells, which express estrogen receptor on the surface, are very sensitive to artemisinin and its derivatives. Due to the influence of the drug in the estrogen-dependent cells, including MCF-7 and T47D, there is inhibition of expression of estrogen receptor α on an epigenetic level by blocking the availability of ESR1 promoter to transcription factors [7].

We and others found that in addition to the inhibition of steroid signaling cascades, artemisinin affects other epigenetic mechanisms regulating the expression of proteins. In particular, Hargraves et al. [18] demonstrated that levels of miR-34a affect the Artemisinin ability to stop the cell cycle of BC cells regardless of *TP53* mutations. As it is known, miR-34a is one of the effectors of p53-mediated apoptosis. Artemisinin significantly increases the levels of miRNAs in MCF-7 and T47D cells but does not affect expression levels of p53 protein [18].

As noted above, one of the mechanisms of cell cycle arrest by artemisinin is CDK4-kinase pathway. CDK4 is a direct target of miR-34a. In addition, expression of this onco-suppressive microRNA affects the sensitivity to a wide range of cytotoxic drugs, including DDP. Given the above, we have elucidated the effect of artemisinin on the expression of miR-34a and a number of other miRNAs, which are involved in the development of BC cell resistance to anticancer drugs. In particular, in previous studies, we found that the development of resistance to DDP and Dox in MCF-7 cells is associated with lower levels of miR-200b, -320a and changes of the expression of miR-34a [19].

MiR-200b and -320a both participate in the regulation of intracellular iron metabolism. The target of miR-200b is a ferritin heavy chain mRNA and the target of miR-320a is mRNA of CD71. For example, increased expression of miR-320a reduces the level of CD71 on the surface of tumor cells and, thereby, reduces the iron absorption [20].

This is expected to affect the sensitivity of tumor cells to artemisinin and its derivatives. We observed that incubation of the MCF-7 cells with different sensitivity to cytotoxic drugs with artemisinin expression of miR-200b, -320a, and -34a changed (Figure). In particular, the levels of miR-34a and -320a increased both in sensitive and resistant to Dox MCF-7 cell lines. At the same time in all studied cells we observed increased levels of miR-200b. These data suggest that the reduced sensitivity of MCF-7/DDP to artemisinin is mediated not only by violations in regulating proteins levels. It can be caused by features of epigenetic regulation of expression of proteins responsible for iron metabolism. There are many reports of selective cytotoxicity of artemisinin and its derivatives on tumor cells of prostate, breast, larynx, blood and other cancers. In addition, there is the evidence that cell death occurs only by apoptosis because artemisinin does not induce necrosis. Necrotic cells release large amounts of cytokines after lysis, thereby inducing inflammation, which is one of the shortcomings of most anticancer drugs [21]. Selective

toxicity artemisinin on tumor cells and its low toxicity on normal breast cells were confirmed in experiments *in vitro* by Singh *et al.* [22]. It is known that only one third of circulating transferrin molecules are loaded with iron and oral iron salts significantly increase the concentration of iron ions in breast tumors. This is due to the fact that BC cells actively absorb iron from the blood. In *in vivo* experiments the possibility of increasing the sensitivity of tumor cells to artemisinin by this way was demonstrated [23].

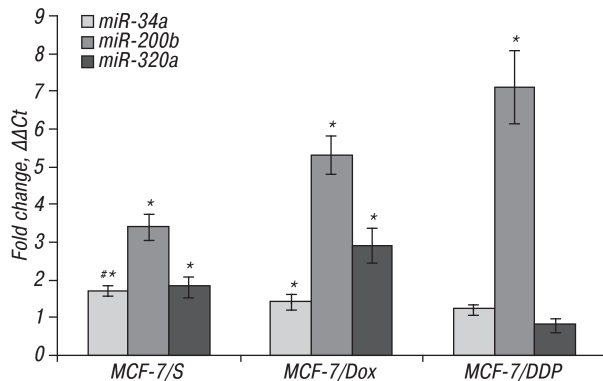

Figure. Relative miR-34a, -200b, and 320a expression in MCF-7 cells with different sensitivity to cytostatic drugs after artemisinin treatment compared to untreated control
Note: #coincides with Hargraves et al. results [19]; $*p \leq 0.05$ compared with cell lines without artemisinin

Obtained data suggest artemisinin cytotoxic effect on BC cells and the ability of the drug to improve the sensitivity of tumor cells to anticancer drugs. In addition, the possibility of correction of therapeutic dose using a magnetic fluid that can be used to increase the sensitivity of transformed cells to the action of cytostatics.

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